

**SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION**  
**GUIDELINES**

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## **SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION**

### **GUIDELINES**

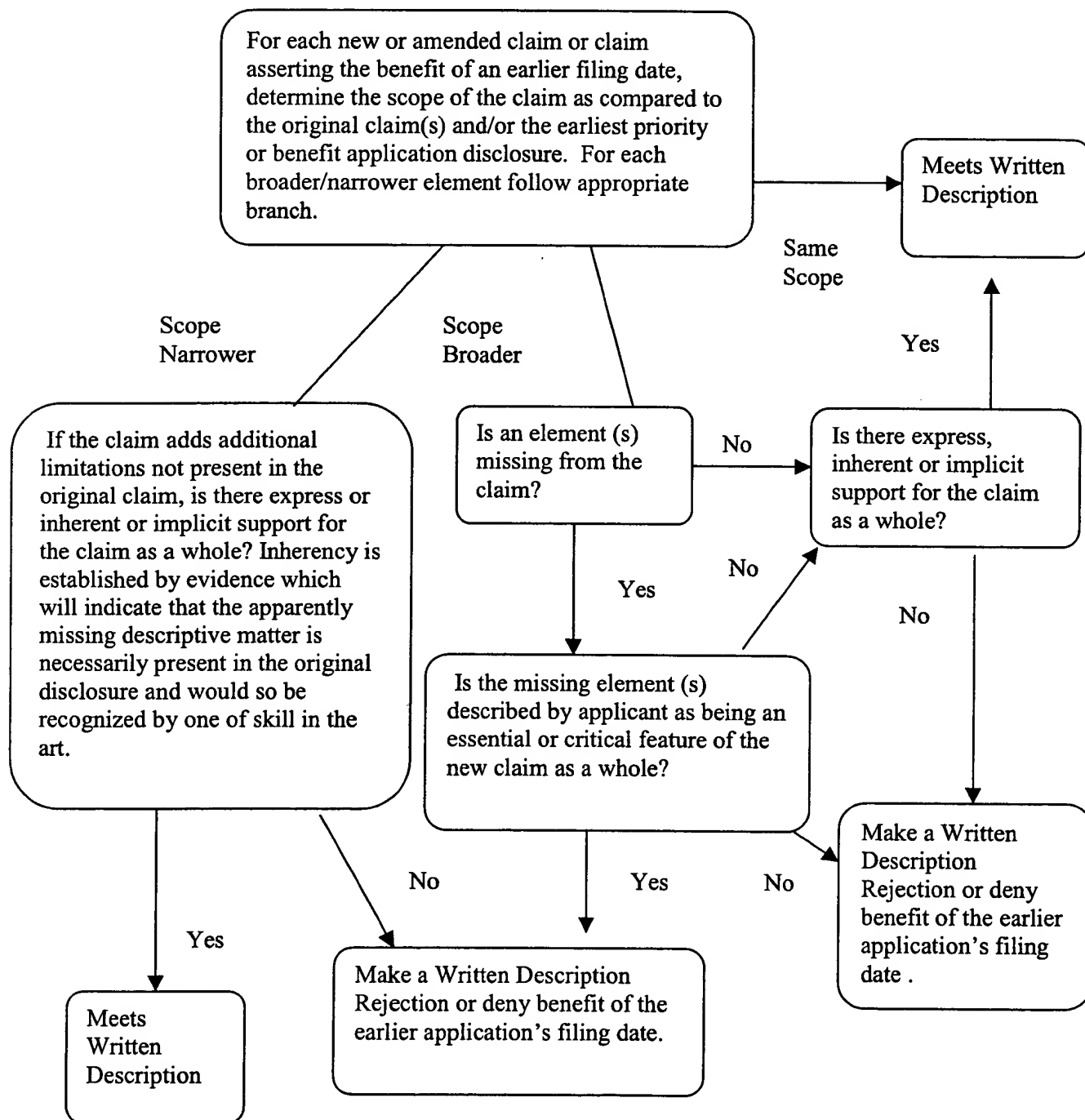
It is assumed at this point in the analysis that the specification has been reviewed and an appropriate search of the claimed subject matter has been conducted. It is also assumed that the examiner has identified which features of the claimed invention are conventional taking into account the body of existing prior art. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. If the examiner determines that the application does not comply with the written description requirement, the examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. It should also be noted that the test for an adequate written description is separate and distinct from the test under the enablement criteria of 35 U.S.C. § 112 first paragraph. The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, para. 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

The following examples only describe how to determine whether the written description requirement of 35 U.S.C. 112, para. 1 is satisfied. Regardless of

the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of Title 35 of the U.S. Code. Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

**Written Description Amended**  
**or New Claims, or Claims Asserting**  
**the Benefit of an Earlier Filing Date**

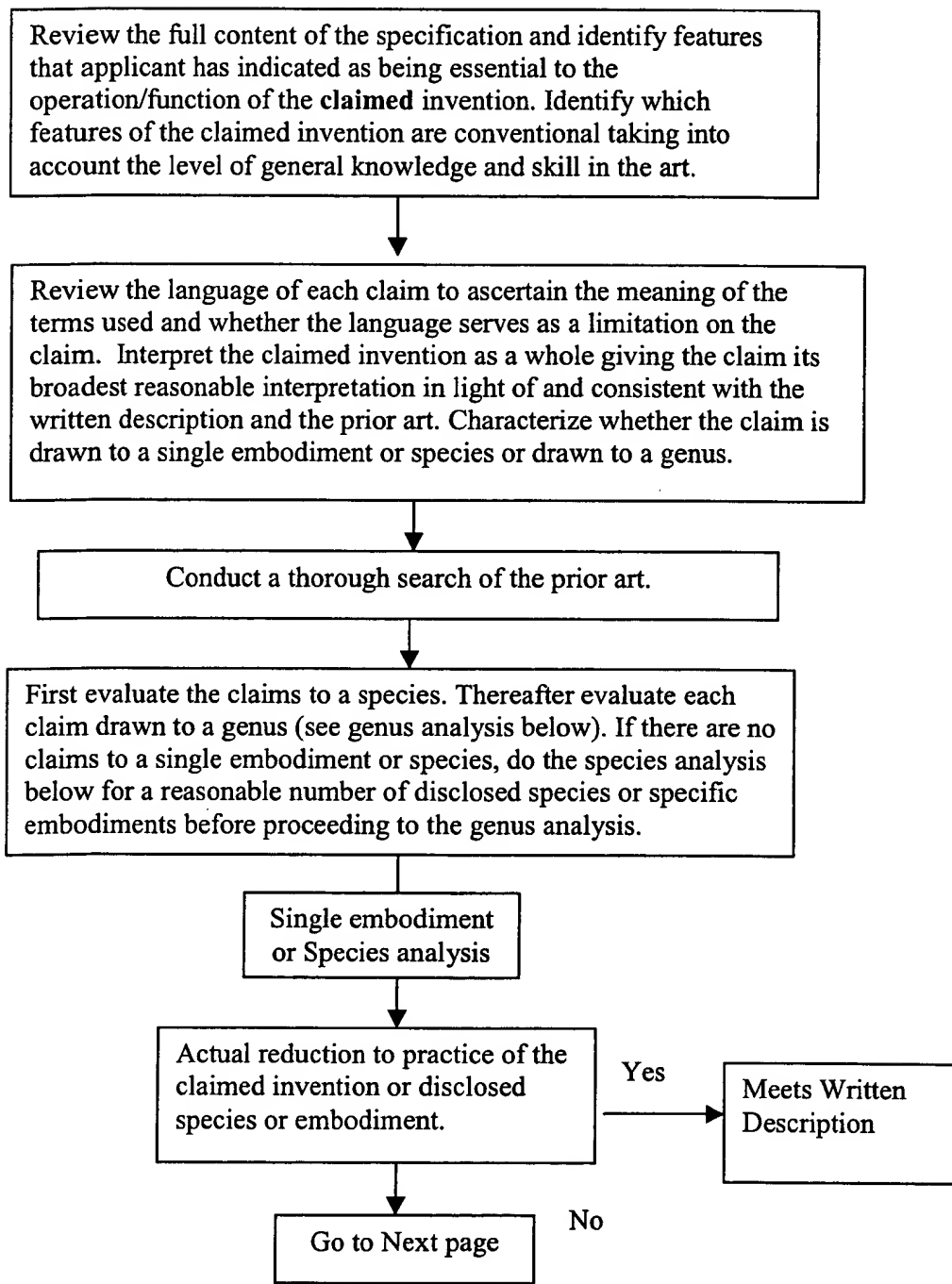
**Decision Tree**



## Written Description

### Original Claims

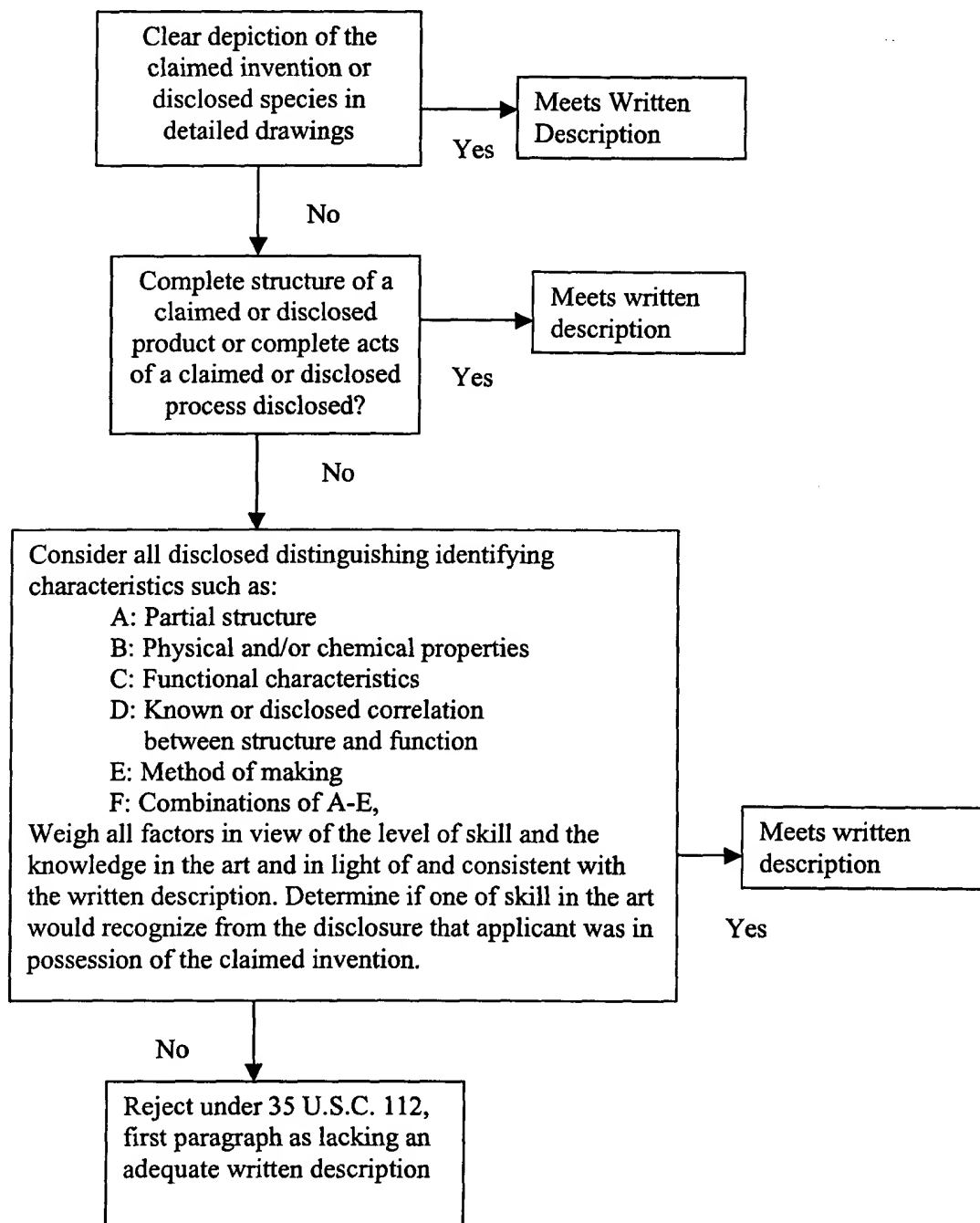
#### --Decision Tree--



Written Description

Original Claims

--Decision Tree--





**Written Description**

**Original Claims**

**Decision Tree**

**--Page 3--**

**Genus Analysis**

Determine whether the art indicates substantial variation among the species within the genus of the claimed subject matter.

Is there is a representative number of species implicitly or explicitly disclosed?  
What is a representative number of species depends on whether one of skill in the art would recognize that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed or claimed.

Yes

**Meets Written Description**

No

Make a rejection under 35 USC 112 first paragraph as lacking written description.

## **WRITTEN DESCRIPTION TRAINING EXAMPLES**

### **Example 1: Amended claims**

#### **Fact Pattern:**

The specification is directed to a sectional sofa with a console between two reclining chairs, wherein control means for the reclining chairs are mounted on the console. The original disclosure clearly identifies the console as the only possible location for the controls, and provides for only the most minor variation in the location of the controls, e.g., the controls may be mounted on the top or side surfaces of the console or on the front wall. Additionally, the specification states that the purpose for the console is to house the controls. The original claims required the control elements to be present in the console. Applicant subsequently amends the claims to remove this limitation.

#### **Amended Claim:**

##### **1. (Amended) A sectional sofa comprising:**

a pair of reclining seats disposed in parallel relationship with one another in a double reclining seat sofa section, said double reclining seat sofa section being without an arm at one end whereby a second sofa section of the sectional sofa can be placed in abutting relationship with the end of the double reclining seat sofa section without an arm so as to form a continuation thereof,

each of said reclining seats having a backrest and seat cushion and movable between upright and reclined positions, said backrests and seat cushions of the pair of reclining sets lying in respective common planes when the seats are in the same positions,

a fixed console disposed in the double reclining seat sofa section between the pair of reclining seats and with the console and reclining seats together comprising a unitary structure, said console including an armrest portion for each of the reclining seats, said arm rests remaining fixed when the reclining seats move from one to another of their positions, and

**a pair of control means [located upon the center console to enable each of the pair of reclining seats to move separately between the reclined and upright positions] mounted on the double reclining seat sofa section and each readily accessible to an occupant of its respective reclining seat and when actuated causing the respective reclining seat to move from the upright to the reclined position.**

**Analysis:**

The amended claim is broader than the original claim in that the pair of control means is no longer required to be located on the center console. Thus, control means mounted on a center console is an element missing from the claim. The specification describes the location of the control means on the console as an essential feature of the claimed invention as a whole because the specification clearly identifies the console as the only possible location for the controls, and states that the purpose for the console is to house the controls.

**Conclusion:**

Reject the amended claim under 35 USC §112 first paragraph as lacking adequate written description.

## **Example 2: 35 USC 120 Priority**

### **Fact Pattern:**

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup is not important, as long as the implant can effectively function as an artificial hip socket. The application is a continuation in part of a parent application that describes an acetabular cup prosthesis wherein the cup is a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The parent specification also touts the criticality of a conical cup over all other shape cups.

A reference disclosing the claimed invention published between the filing date of the parent application and the instant application. Applicant asserts entitlement to the filing date of the parent application.

### **Claim:**

1. An acetabular cup prosthesis comprising (1) a body extending generally longitudinally and terminating into front and rear surfaces, said front surface extending substantially transversely to said body; and (2) at least one fin for securing said cup to a prepared acetabulum cavity, said fin having a length extending generally longitudinally from said front surface toward said rear surface continuously along said body throughout the entire length of said fin, and said fin being configured so as to extend radially outwardly beyond the perimeter of said front surface and said body so as to engage with the cavity thereby securing said cup.

2. The prosthesis of claim 1, wherein the body has a generally conical outer surface.

**Analysis:**

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the parent application, which only describes a conical cup. Claim 1 is missing the element of a conical shape. This element is an essential or critical feature of the invention described in the parent application because the parent application only discloses a conical shape and the conical shape is described as critical over other shapes.

Claim 2 of the instant application is directed to an acetabular cup prosthesis wherein the cup has a generally conical outer surface. The claim is of the same scope as the invention described in the parent application.

**Conclusion:**

Reject claim 1 over the prior art reference, and indicate that the claim is not entitled to the benefit of the earlier application filing date.

Indicate that claim 2 is entitled to the benefit of the parent application filing date.

Note that if applicant had added the subject matter of claim 1 of this application to the parent application in an amendment, the claim would have been rejected under 35 U.S.C. 112, first paragraph as lacking an adequate written description.

**Example 2A: Essential element missing from original claim**

**Fact Pattern:**

The fact situation of example 2 above is similar to the fact situation of the instant example, however, there is no parent application in this example.

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup is critical to permit the implant to effectively function as an artificial hip socket. The application describes an acetabular cup prosthesis wherein the cup is a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The specification also touts the criticality of a conical cup.

**Claims:** Same as claims 1 and 2 of example 2 above.

**Analysis:**

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the instant application that only describes a conical cup. Claim 1 is missing the element of a conical shape. A review of the specification indicates that a cup implant having a shape which can effectively function as an artificial hip socket is critical to the operation/function of the claimed invention. The application discloses a conical shape cup and the conical shape is described as critical over other shapes. The specification indicates that the invention **as claimed** will not function in its intended manner without the specific cup

shape. Therefore this element is essential to the function/operation of the invention.

Claim 1 is directed to a genus. There is no actual reduction to practice or clear depiction of the claimed invention in detailed drawings; however, the complete structure of a species of the claimed prosthesis (with conical shape) is disclosed. The disclosed species is not representative of the genus because the specification indicates that without the conical shape the invention will not operate as intended. Therefore, applicant was not in possession of the necessary common attributes of the elements possessed by the members of the genus. A written description rejection should be made in this situation.



**Example 2B: A preferred element missing from original claim**

**Fact Pattern:**

The fact situation of example 2B is similar to example 2A above except that in this example the shape of the conical cup is described as being preferred.

The specification is directed to artificial hip sockets that include cup implants adapted for insertion into an acetabular, or hip, bone. The specification indicates that the shape of the cup must permit the implant to effectively function as an artificial hip socket. The application describes an acetabular cup prosthesis wherein the cup is preferably a trapezoid, a truncated cone, or of conical shape. All of these terms describe a conical cup. The specification emphasizes that a conical cup is the preferred embodiment.

**Claims:** Same as claims 1 and 2 of example 2 above.

**Analysis:**

Claim 1 in the instant application is directed to an acetabular cup prosthesis wherein the shape of the cup is not specifically defined (see element (1) of claim 1). The claim is broader than the disclosure in the instant application that only describes a conical cup. Claim 1 is missing the element of a conical shape. A review of the specification indicates that a cup implant having a conical shape is preferred but has no apparent bearing to the operation/function of the claimed invention. Therefore this element is not essential to the function or operation of the invention.

Claim 1 is directed to a genus. Although there is no actual reduction to practice or clear depiction of the claimed invention in detailed drawings, the complete structure of a species of the claimed prosthesis (with conical shape) is disclosed. The disclosed species is representative of the genus because there is a known correlation between the structure and the function of claimed invention and one of skill in the art would recognize that applicant was in possession of the necessary common attributes of the elements possessed by the members of the genus. The invention as claimed will function in its intended manner even without the specific cup shape. No written description rejection should be made in this situation.

**Note: If the specification needs to be amended to be consistent with an original claim, see MPEP 608.01(o).**

### **Example 3: New claims**

#### **Fact Pattern:**

The specification describes a form of computer technology called multi-threading. In essence, computers with multi-threading capabilities can switch between tasks with such rapidity that they appear to be performing two or more tasks at once. The specification describes one illustrative example in the specification wherein one of the program threads is an editor and another thread is a code processing routine in the form of a compiler. As the operator strikes keys at the keyboard, the compiler thread executes between each successive pair of keystrokes to process the entered source code concurrently with the editing operation. By the time the operator has finished entering or editing the code the compiler thread will have completed most of the required processing, thereby freeing the operator from lengthy periods of waiting for extensive code processing.

In this illustrative embodiment the interrupt operation of the central processor is periodically activated by a timer or clock. Each interrupt operation asynchronously preempts the executing compiler thread and passes control of the central processor to an interrupt service routine. The input port is then polled to test if a key has been struck at the keyboard. If not, the interrupt is terminated and control returns to the compiler thread. If polling the port reveals that a key has been struck then the interrupt service routine invokes the editor thread which takes control of the central processor to perform a character code entry or other edit operation. In addition to the description above, the application's abstract references an editor, compiler, interrupt means, and return means, and the "Object of the Invention" section

and the "Description of Prior Art" clearly discuss the importance of an editor and compiler.

The original claims required, *inter alia*, an editor, a compiler, an interrupt means and a return means. These elements are missing from new claim 20.

**Claim:**

20. A computer-readable disk memory having a surface formed with a plurality of binary patterns constituting a multithreaded application program executable by a desktop computer having a central microprocessor, a memory, means for loading said application program into a defined address space of said memory, and a clock-driven periodically-activated interrupt operation, said multithreaded program comprising

a plurality of sets of instructions with each set executable by said microprocessor,

a first of said sets of instructions executable to provide a first thread of execution having control of the central microprocessor,

said first thread of execution being periodically preempted in response to activations of an interrupt operation at predetermined fixed time intervals, and

a second of said sets of instructions executable to provide a second thread of execution to acquire control of the central microprocessor,

each of said threads having direct access to said program memory address space so as to provide fast efficient preemption of one thread by

another thread and switching of control of the central microprocessor back and forth among the threads at a rate so rapid that the threads execute effectively simultaneously.

**Analysis:**

Claim 20 is a new claim, which is broader in scope than the original claims. There are four elements missing from the claims (the editor, compiler, interrupt means, and return means). These missing elements are described by applicant as being an essential or critical feature of the claimed invention as a whole as evidenced by applicant's repeated reliance on the presence of these elements throughout the originally filed disclosure. Multiple sections within the application make clear that these four elements served integral functions in the overall invention.

**Conclusion:**

Reject claim 20 as lacking an adequate written description because four elements described as essential or critical are omitted. The omitted elements are: editor, compiler, interrupt means, and return means.

#### **Example 4 : Original claim**

##### **Fact Pattern:**

The invention is directed to a form of autopilot, described as a "heading lock," which enables a person to maintain directional control over a watercraft without constant manipulation of trolling motor controls. The preferred embodiment, as set forth in the written description and clearly depicted in detailed drawings, employs a compass mounted to the head of the "heading lock" unit, which monitors the direction of the thrust motor. The heading lock is coupled to the trolling motor; in a preferred embodiment, the heading lock is mechanically coupled to the trolling motor. The disclosure specifically notes that the direction of the thrust motor is considered to be the same as the direction of the boat since the trolling motor is mounted on the bow of the boat. The specification indicates that the electronic steering system continues to monitor the current heading of the thrust and also indicates that the heading detector continuously monitors the current heading of the boat. The term "heading" is used interchangeably throughout the written description to refer to both the direction of the trolling motor and the direction of the boat.

##### **Claim:**

1. A heading lock coupled to a trolling motor producing a thrust disposed to pull a watercraft, said heading lock comprising:

a steering motor coupled to said trolling motor, said steering motor being disposed to affect the orientation of said trolling motor in response to input signals;

a steering circuit electrically coupled to said steering motor, said steering circuit being disposed to generate said input signals to said steering motor in response to heading signals; and

a heading detector electrically coupled to said steering circuit, said heading detector being disposed to transmit said heading signals to said steering circuit.

**Analysis:**

Applicant has identified a heading lock comprising a steering system coupled to a trolling motor and a heading detector, as features essential to the operation of the claimed invention. Although the heading lock is preferably mechanically coupled to the trolling motor, the applicant does not describe the type of coupling as essential to the claimed invention as a whole. A search of the prior art shows that various means for coupling a heading lock to a trolling motor are conventional in the art. The claim is drawn to a single embodiment. Although there is no reduction to practice of the claimed invention, the claimed invention is clearly depicted in detailed drawings.

**Conclusion:**

The claim is adequately described.

### **Example 5: Flow Diagrams**

#### **Fact Pattern:**

The specification is directed to a mechanism for controlling the mode of operation of a modem. A modem is used for modulating and demodulating signals, both analog and digital, over telephone lines. It has two modes: (1) a transparent mode, in which the modem performs the modulation-demodulation function, and (2) a command mode, in which the modem responds to predetermined commands and performs operations by executing a set of instructions stored in Read-Only-Memory (ROM) or firmware. An escape command tells the modem when to switch between transparent and command modes.

The application claims an improved mechanism for detecting an escape command by a modem. The decision making capability and timing means preferably reside in a microprocessor, preferably a Z-8 type microprocessor. The specification discloses logic flow diagrams and provides a detailed functional recitation that describes how to program computers to detect an escape command, but the specification does not provide a computer program listing with source code. The specification describes the escape sequence as one full second of no data, followed by the predetermined escape command, followed by another full second of no data.

#### **Claim:**

1. In a modem including a data input port for connecting said modem to a utilization device, and a telephone port for connecting said modem to a



telephone line, said modem being of the type having two distinct modes of operation:

(a) a transparent mode of operation for which said modem provides modulated signals to said telephone port in response to data signals provided to said data input port; and

(b) a command mode of operation for which said modem responds to said data signals provided to said data input port as instructions to said modem;

said modem including means defining a predetermined sequence of said data signals as an escape character; the improvement comprising:

timing means for detecting each occurrence of a passage of a predetermined period of time after provision of one of said data signals to said data input port; and

means, operative when said modem is in said transparent mode of operation, for detecting provision of said predetermined sequence of said data signals, and for causing said modem to switch to said command mode of operation, if and only if said predetermined sequence of data signals occurs contiguous in time with at least one said occurrence of said passage of said predetermined period of time during which none of said data signals are provided to said data input port.

#### **Analysis:**

After a review of the full content of the specification, the examiner finds that a modem having two modes of operation (transparent and

command), a timing means, and a means for detecting an escape sequence and causing the modem to switch from the transparent to the command mode are essential to the operation and function of the claimed invention. The specification does not describe a particular timing means or means for detecting the escape command and switching to the command mode. The claim is drawn to a genus. A search of the prior art indicates that the structure of the hardware required is conventional, and that one skilled in the art would know how to program a microprocessor to perform the necessary steps described in the specification. A review of the art indicates that there is no substantial variation among the species within the genus. Although no embodiments have been actually reduced to practice, a review of the specification shows that the claimed invention has been reduced to drawings in view of the detailed functional flow diagrams. Since the claimed invention is supported by conventional hardware structure and because there is a functional description of what the software does to operate the computer, there is sufficient description of the claimed invention. Disclosing a microprocessor capable of performing certain functions is sufficient to satisfy the requirement of section 112, first paragraph, when one skilled in the relevant art would understand what is intended and know how to carry it out.

**Conclusion:**

The claimed invention has been adequately described.

## **Biotechnology Examples**

### **Example 6: Genes**

**Specification:** The specification describes an isolated cDNA fragment (SEQ ID NO: 1; a 100mer) obtained from a human glioblastoma cDNA library. SEQ ID NO: 1 is asserted to be homologous to a known DNA molecule that encodes the extracellular domain of a glial specific G-coupled protein receptor whose function is associated with glial cell differentiation. The observed homology is sufficient to support a conclusion that SEQ ID NO: 1 would be glial specific. Further, it would be reasonable to infer that a G-coupled protein receptor encoded by a cDNA that comprised SEQ ID NO: 1 would be involved in the regulation of glial cell differentiation. In the description, applicant defines a “gene” as including naturally occurring regulatory elements and untranslated regions necessary and sufficient to mediate the expression of a cDNA comprising SEQ ID NO: 1. The specification describes methods for cloning nucleic acids that encode full-length glial specific G coupled protein receptors. The specification also discloses that SEQ ID NO: 1 can be used as a probe for identifying the presence of nucleic acids encoding glial specific G-coupled protein receptors in mammals. Glial specific G-coupled protein receptors are disclosed as useful in drug discovery methods to identify agents that regulate glial differentiation. The specification defines a probe as consisting of SEQ ID NO: 1 and between five to 10 additional nucleotides on either end of SEQ ID NO: 1.

**Claim:**

An isolated gene comprising SEQ ID NO: 1.

**Analysis:**

A review of the specification indicates that elements which are not particularly described, including regulatory elements and untranslated regions, are essential to the function of the claimed invention because applicant's definition of "gene" requires them. Additionally, SEQ ID NO: 1 is disclosed as being essential to the function of the claimed invention. The art indicates that the structure of genes with naturally occurring regulatory elements and untranslated regions is empirically determined. For example, the structural elements of "gene" mediating the expression of a particular protein in the liver may be different than the structural elements of the "gene" mediating the expression of the same protein in the brain. Therefore the structure of these elements which applicant considers as being essential to the function of the claim are not conventional in the art.

The claim is drawn to a genus, i.e., any gene which comprises SEQ ID NO: 1.

A search of the prior art indicates that SEQ ID NO: 1 is otherwise novel and unobvious, and no associated genomic clones have been identified.

There is no actual reduction to practice of the claimed invention, clear depiction of the claimed invention in the drawings or complete detailed description of the structure.

Considering all disclosed distinguishing identifying characteristics, there is a disclosure of partial structure (SEQ ID NO: 1) as well as the function of the gene as coding for a G-coupled protein receptor.

However, there is no known or disclosed correlation between this function and the structure of the non-described regulatory elements and untranslated regions of the gene. Furthermore, there is no additional disclosure of physical and/or chemical properties. Weighing all factors in view of the level of knowledge and skill in the art, one skilled in the art would not recognize from the disclosure that the applicant was in possession of the genus of genes which comprise SEQ ID NO: 1.

**Conclusion:**

Reject claim 1 under 35 USC 112 first paragraph as lacking an adequate written description. The examiner should make a rejection following a similar type of reasoning as that set forth above.

**Note: Applicant may overcome this rejection by claiming a probe which consists essentially of SEQ ID NO: 1, since the specification teaches that a probe can have no more than 10 additional nucleic acid residues at either end of the molecule. The examiner should make an express determination that “consisting essentially of” admits of no more than 10 additional residues at either end of the molecule.**

### **Example 7: EST**

**Specification:** The specification discloses SEQ ID NO: 16 which is a partial cDNA. The specification does not address whether the cDNA crosses an exon/intron splice junction. The specification discloses that this sequence will specifically hybridize with the complement of the coding sequence of a gene of an infectious yeast. The presence of the nucleic acid detected by hybridization with the complement of the coding sequence is useful for identifying yeast infections. Example 1 of the specification describes an experiment where SEQ ID NO: 16 was determined following characterization of a cDNA clone isolated from a cDNA library.

#### **Claim:**

An isolated DNA comprising SEQ ID NO: 16.

#### **Analysis:**

A review of the full content of the specification indicates SEQ ID NO: 16 is essential to the operation and function of the claimed invention. The specification indicates that the presence of DNA that hybridizes with SEQ ID NO: 16 is indicative of a yeast infection.

A review of the language of the claim indicates that the claim is drawn to a genus, i.e., any nucleic acid that minimally contains SEQ ID NO: 16 within it including any full length gene which contains the sequence, any fusion constructs or cDNAs.

The search indicates that SEQ ID NO: 16 is a novel and unobvious sequence.

There is a single species explicitly disclosed (a molecule consisting of SEQ ID NO: 16 that is within the scope of the claimed genus).

There is actual reduction to practice of the disclosed species.

The disclosure of a single disclosed species may provide an adequate written description of a genus when the species disclosed is representative of the genus. The present claim encompasses full-length genes and cDNAs that are not further described. There is substantial variability among the species of DNAs encompassed within the scope of the claims because SEQ ID NO: 16 is only a fragment of any full-length gene or cDNA species. When reviewing a claim that encompasses a widely varying genus, the examiner must evaluate any necessary common attributes or features. In the case of a partial cDNA sequence that is claimed with open language (comprising), the genus of, e.g., “A cDNA comprising [a partial sequence],” encompasses a variety of subgenera with widely varying attributes. For example, a cDNA’s principle attribute would include its coding region. A partial cDNA that did not include a disclosure of any open reading frame (ORF) of which it would be a part, would not be representative of the genus of cDNAs because no information regarding the coding capacity of any cDNA molecule would be disclosed. Further, defining “the” cDNA in functional terms would not suffice in the absence of a disclosure of structural features or elements of a cDNA that would encode a protein having a stated function.

A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a

substantial portion of the genus. Regents of the University of California v. Eli Lilly & Co., 119 F3d 1559, 1569, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

Here, the specification discloses only a single common structural feature shared by members of the claimed genus, i.e., SEQ ID NO: 16. Since the claimed genus encompasses genes yet to be discovered, DNA constructs that encode fusion proteins, etc., the disclosed structural feature does not "constitute a substantial portion" of the claimed genus. Therefore, the disclosure of SEQ ID NO: 16 does not provide an adequate description of the claimed genus.

Weighing all factors, 1) partial structure of the DNAs that comprise SEQ ID NO: 16, 2) the breadth of the claim as reading on genes yet to be discovered in addition to numerous fusion constructs and cDNAs, 3) the lack of correlation between the structure and the function of the genes and/or fusion constructs; in view of the level of knowledge and skill in the art, one skilled in the art would not recognize from the disclosure that the applicant was in possession of the genus of DNAs which comprise SEQ ID NO: 16.

**Conclusion:** The written description requirement is not satisfied.



**Caveat:** *In situations where the specification indicates that the SEQ ID NO: is a full-length cDNA open reading frame and the claim cannot read on a gene, the claimed invention would meet the written description requirement.*

**Example 8: DNA fragment Encoding a Full Open Reading Frame (ORF)**

**Specification:** The specification discloses that a cDNA library was prepared from human kidney epithelial cells and 5000 members of this library were sequenced and open reading frames were identified. The specification discloses a Table that indicates that one member of the library having SEQ ID NO: 2 has a high level of homology to a DNA ligase. The specification teaches that this complete ORF (SEQ ID NO: 2) encodes SEQ ID NO: 3. An alignment of SEQ ID NO: 3 with known amino acid sequences of DNA ligases indicates that there is a high level of sequence conservation between the various known ligases. The overall level of sequence similarity between SEQ ID NO: 3 and the consensus sequence of the known DNA ligases that are presented in the specification reveals a similarity score of 95%. A search of the prior art confirms that SEQ ID NO: 2 has high homology to DNA ligase encoding nucleic acids and that the next highest level of homology is to alpha-actin. However, the latter homology is only 50%. Based on the sequence homologies, the specification asserts that SEQ ID NO: 2 encodes a ligase.

**Claim 1:** An isolated and purified nucleic acid comprising SEQ ID NO: 2.

**Analysis:**

A review of the full content of the specification indicates SEQ ID NO: 2 is essential to the operation and function of the claimed invention. The specification indicates that SEQ ID NO: 2 encodes a protein that would be expected to act as a DNA ligase.

A review of the language of the claim indicates that the claim is drawn to a genus, i.e., any nucleic acid that minimally contains SEQ ID NO: 2. The claim is drawn to a nucleic acid comprising a full open reading frame. The claimed nucleic acid does not read on a genomic sequence because full-length mammalian cDNAs would not be expected to contain introns or transcriptional regulatory elements such as promoters that are found in genomic DNA. The claim reads on the claimed ORF in any construct or with additional nucleic acid residues placed at either end of the ORF.

The search indicates that SEQ ID NO: 2 is a novel and unobvious sequence.

There is a single species explicitly disclosed (a molecule consisting of SEQ ID NO: 2 that is within the scope of the claimed genus).

There is actual reduction to practice of the disclosed species.

One of skill in the art can readily envisage nucleic acid sequences which include SEQ ID NO: 2 because e.g. SEQ ID NO: 2 can be readily embedded in known vectors. Although there may be substantial variability among the species of DNAs encompassed within the scope of the claim because SEQ ID NO: 2 may be combined with sequences known in the art,

e.g. expression vectors, the necessary common attribute is the ORF (SEQ ID NO: 2).

Weighing all factors including (1) that the full length ORF (SEQ ID NO: 2) is disclosed and (2) that any substantial variability within the genus arises due to addition of elements that are not part of the inventor's particular contribution, taken in view of the level of knowledge and skill in the art, one skilled in the art would recognize from the disclosure that the applicant was in possession of the genus of DNAs that comprise SEQ ID NO: 2.

**Conclusion:** The written description requirement is satisfied.

**Example 9: Hybridization**

**Specification:** The specification discloses a single cDNA ( SEQ ID NO:1) which encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. The specification includes an example wherein the complement of SEQ ID NO: 1 was used under highly stringent hybridization conditions (6XSSC and 65 degrees Celsius) for the isolation of nucleic acids that encode proteins that bind to dopamine receptor and stimulate adenylate cyclase activity. The hybridizing nucleic acids were not sequenced. They were expressed and several were shown to encode proteins that bind to a dopamine receptor and stimulate adenylate cyclase activity. These sequences may or may not be the same as SEQ ID NO: 1.

**Claim:**

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1,

wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

**Analysis:**

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus of nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity.

The search of the prior art indicates that SEQ ID NO: 1 is novel and unobvious.

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of

skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

**Conclusion:** The claimed invention is adequately described.

**Example 10: Process claim**

**Specification:** The specification teaches that SEQ ID NO: 10 is an EST. The specification also teaches that SEQ ID NO: 10 is a chromosome marker and that any DNA which hybridizes under specified stringent conditions to SEQ ID NO: 10 will be useful as a marker for detecting the presence of Burkitt's lymphoma. The specification also teaches how to produce DNAs including genomic DNAs which hybridize to SEQ ID NO: 10 and isolation of said DNAs. The specification presents an example where a genomic DNA is probed with SEQ ID NO: 10 under the specified stringent conditions (6XSSC and 65 degrees Celsius) and the genomic DNA which hybridizes under these conditions is isolated and is sequenced. The sequence of this genomic clone is represented by SEQ ID NO: 11.

**Claim:**

Claim 1: A process for producing an isolated polynucleotide comprising hybridizing SEQ ID NO: 10 to genomic DNA in 6XSSC and 65° C and isolating the DNA polynucleotide detected with SEQ ID NO: 10.

Claim 2: An isolated DNA that hybridizes with SEQ ID NO: 10.

**Analysis:****Claim 1:**

A review of the full content of the specification indicates that the essential feature of the claimed invention is a process of obtaining a nucleic acid sequence which is identified by a probe that hybridizes to SEQ ID NO:10 and a polynucleotide that hybridizes with SEQ ID NO: 10. The

specification and the general state of the art indicate that the general process of producing nucleic acids through hybridization with probes was routine at the time of filing.

The claim is drawn to a genus i.e., a process of hybridizing to genomic DNA with SEQ ID NO: 10 and isolating the DNA which hybridizes under specific conditions to said sequence.

The search indicates that SEQ ID NO: 10 and SEQ ID NO: 11 are novel and unobvious sequences. Therefore, under the examination guidelines of *In re Ochiai* and *In re Brouwer*, the method of making a novel and unobvious product is also novel and unobvious.

The specification presents an example where a single species has been reduced to practice, i.e., isolation of SEQ ID NO: 11 based on hybridization with SEQ ID NO: 10. Therefore the disclosed species within the genus has been adequately described. Now turning to the genus analysis, the art indicates that there is no substantial variation within the genus because of the stringency of hybridization conditions which yields structurally similar molecules. The single disclosed species is representative of the genus because reduction to practice of this species, considered along with the defined hybridization conditions and the level of skill and knowledge in the art, are sufficient to allow the skilled artisan to recognize that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus.

**Claim 2:**

The claim is drawn to a genus of nucleic acids, all of which must hybridize to SEQ ID NO: 10. The claim does not specify any stringency conditions. The claim is broad and reads on virtually any nucleic acid.

There is a species disclosed, SEQ ID NO: 11. The art indicates that there is substantial variation within the genus because the lack of stringency of hybridization conditions would be expected to yield structurally unrelated nucleic acid molecules. The single disclosed species is not representative of the genus because there is no structural attribute or feature that is common to the members of the genus.

**Conclusion:**

Claim 1 is adequately described.

Claim 2 should be rejected as lacking adequate written description following the analysis described above.

**Note: Applicant may overcome the written description rejection of the product by, for example, substituting claim 2 with a product by process claim such as the one below.**

*Claim 2. The isolated DNA polynucleotide prepared according to the process of claim 1.*



**Example 11: Allelic Variants**

**Specification:** The specification discloses a DNA, SEQ ID NO: 1, said to encode a cell surface receptor for adenovirus. The cell surface receptor is designated protein X and its sequence is given as SEQ ID NO:2. The specification states that the invention includes alleles of the DNA that include single nucleotide polymorphisms (SNPs). No allelic sequence information is disclosed, but the specification states that allelic variants of SEQ ID NO: 1 can be obtained, e.g., by hybridizing SEQ ID NO: 1 to a DNA library made from the species of organism that yielded SEQ ID NO: 1.

**Claims:**

1. An isolated DNA that encodes protein X (SEQ ID NO: 2).
2. An isolated allele of the DNA according to claim 1, which allele encodes protein X (SEQ ID NO: 2).
3. An isolated allele of SEQ ID NO: 1.

**Analysis:****Claim 1:**

Claim 1 is drawn to the genus of DNAs that encode amino acid sequence SEQ ID NO:2, i.e., all sequences degenerately related by a genetic code table to SEQ ID NO:1. Although only one specie within the genus is disclosed, SEQ ID NO:1, a person of skill in the art could readily envision all the DNAs degenerate to SEQ ID NO:1 by using a genetic code table. One of skill in the art would conclude that applicant was in possession of the

genus based on the specification and the general knowledge in the art concerning a genetic coding table.

**Claim 2:**

Claim 2 is drawn to a subgenus of allelic DNAs that encode amino acid sequence SEQ ID NO: 2. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The alleles in claim 2 are "strictly neutral" because they encode identical proteins, and make no difference to phenotype. See, Rieger et al., p. 17. Although the standard definition refers to genomic sequences and the claims are directed to DNAs, a reasonable interpretation is that the claim is directed to DNAs that include naturally occurring mutational site(s).

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of any strictly neutral alleles. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of unknown alleles. The nature of alleles is that they are variant structures, and in the present state of the art the structure of one does

not provide guidance to the structure of others. The common attributes of the genus are not described. One of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

**Claim 3:**

Claim 3 is drawn to the genus including all DNA alleles of SEQ ID NO: 1. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The Rieger reference discloses that there are at least seven different kinds of allele in addition to the “strictly neutral” type discussed above for Claim 2. See, Rieger, pp. 16-17 (amorphs, hypomorphs, hypermorphs, antimorphs, neomorphs, isoalleles, and unstable alleles). The alleles are distinguished by the effect their different structures have on phenotype. According to Rieger, alleles may differ functionally according to their distinct structures. For example, they may differ in the amount of biological activity the protein product may have, may differ in the amount of protein produced, and may even differ in the kind of activity the protein product will have.

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other

members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of different alleles. In addition, according to the standard definition, the genus includes members that would be expected to have widely divergent functional properties. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of other unknown alleles having concordant or discordant functions. The common attributes of the genus are not described and the identifying attributes of individual alleles, other than SEQ ID NO:1, are not described. The nature of alleles is that they are variant structures where the structure and function of one does not provide guidance to the structure and function of others. According to these facts, one of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

### **Conclusions:**

#### **Claim 1:**

Claim 1 should not be rejected under the written description requirement.

#### **Claim 2:**

Claim 2 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see

MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

**Claim 3:**

Claim 3 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

For the rejections of claims 2 and 3, the Office interpretation of "allele" should be supported by a reference, rather than by taking "notice," because the interpretation is the principle evidence supporting the rejection. See MPEP 2144.03 (For further views on official notice, see *In re Ahlert*, 424 F.2d 1088, 1091 165 USPQ 418, 420 - 421 (CCPA 1970) ("[A]ssertions of technical facts in areas of esoteric technology must always be supported by citation of some reference work" and "allegations concerning specific 'knowledge' of the prior art, which might be peculiar to a particular art should also be supported." Furthermore the applicant must be given the opportunity to challenge the correctness of such assertions and allegations. "The facts so noticed serve to 'fill the gaps' which might exist in the evidentiary showing" and should not comprise the principle evidence upon which a rejection is based.); see also, *In re Barr*, 444 F.2d 588, 170 USPQ 330 (CCPA 1971) (scientific journal references were not used as a basis for taking judicial notice that controverted phrases were art - recognized because the court was not sure that the meaning of the term at issue was indisputable among reasonable men); *In re Eynde*, 480 F.2d 470, 178 USPQ

470,474 (CCPA 1973) ("The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are not amenable to the taking of [judicial] notice.".)

### **Example 12: Bioinformatics**

**Specification:** The specification discloses a process for identifying and selecting biological compounds that are present in a biological system in a tissue specific manner. In the disclosed process the expression level of a set of compounds is quantitatively determined in multiple tissues within an organism. The expression level data is then graphically displayed in such a manner that compounds that are differentially expressed are easily identified. An artisan interested in identifying a compound that is expressed at a high level in one tissue and at a different level in a second tissue may easily select compounds that are expressed in a tissue specific manner based on the displayed information. The specification indicates that the compounds to be detected encompass DNA, RNA and proteins as well as metabolites. The specification does not provide any particular examples, but discloses that the expression levels can be determined by any analytical method consistent with the class of compounds being detected. This type of measurement requires actual physical steps.

#### **Claim:**

A computer-implemented method of selecting tissue specific compounds, said method comprising the steps of:

- (a) analyzing the expression level of compounds in a first and second tissue and obtaining expression level data for each of said compounds;
- (b) inputting the expression level data obtained in step a) into a computer;

- (c) displaying a first axis corresponding to the expression level of each of said compounds in said first tissue;
- (d) displaying a second axis substantially perpendicular to said first axis, said second axis corresponding to the expression level data of each of said compound in said second sample
- (e) displaying a mark at a position, wherein said position is selected relative to said first axis in accordance with an expression level of each of said compound in said first sample and relative to said second axis in accordance with the expression of said compound in said second sample; and
- (f) selecting a compound of interest based on the position of the mark.

**Analysis:**

A review of the full content of the specification indicates that obtaining, inputting, and displaying the expression level of compounds is essential to the operation of the claimed invention.

A search of the prior art indicates that obtaining the expression level data of compounds is conventional in the art, and that data display devices and associated support algorithms are well known in the art.

A review of the claim indicates that the claim is drawn to a generic environment for the display of compounds in a tissue specific manner.

Since there is no species claimed or disclosed, the claim is analyzed as a claim drawn to a single embodiment. There is no actual reduction to practice of the claimed invention, or clear depiction of the claimed invention



in detailed drawings. However, reading the specification in light of the knowledge and level of skill in the art, the specification discloses the complete steps of the claimed process. See In re Hayes Microcomputer Products Inc. Patent Litigation, 982 F2d. 1527, 1534-35, 25 USPQ2d 1241, 1246 (Fed. Cir. 1992), where the court stated,

One skilled in the art would know how to program a microprocessor to perform the necessary steps desired in the specification. Thus, an inventor is not required to describe every detail of his invention. An applicant's disclosure obligation varies according to the art to which the invention pertains.

In this fact situation, the art is sufficiently developed so as to put one of skill in the art in possession of the complete steps of the process. In other words, one skilled in the relevant art would understand what is intended by the claimed invention and know how to carry it out.

**Conclusion:** There is adequate written description for what is claimed.

### **Example 13: Protein Variant**

**Specification:** The specification describes a protein isolated from liver. A working example shows that the isolated protein was sequenced and determined to consist of SEQ ID NO: 3. The isolated protein was additionally characterized as being 65 kD in molecular weight and having tumor necrosis activity. The specification states that the invention provides variants of SEQ ID NO: 3 having one or more amino acid substitutions, deletions, insertions and/or additions. No further description of the variants is provided. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and/or additions are routine in the art. The specification does not define when a protein ceases to be a variant of SEQ ID NO: 3.

#### **Claims:**

1. An isolated protein having SEQ ID NO: 3.
2. An isolated variant of the protein of claim 1.

#### **Analysis:**

##### **Claim 1:**

A search of the prior art indicates that SEQ ID NO: 3 is novel and nonobvious. The claim is directed to a genus of proteins that comprise SEQ ID NO: 3. One member of the genus, SEQ ID NO: 3, is described by a complete structure.

There is relatively little variation among the species within the genus because each member of the genus shares SEQ ID NO: 3 as a necessary common feature. The single disclosed example is representative of the claimed genus because taken in view of the general knowledge in the art, the disclosure is sufficient to show that one of skill in the art would conclude that applicant was in possession of the claimed genus.

**Claim 2:**

This is a genus claim. According to the specification, the term variant means a protein having one or more amino acid substitutions, deletions, insertions and/or additions made to SEQ ID NO: 3. The specification and claim do not indicate what distinguishing attributes shared by the members of the genus. The specification and claim do not place any limit on the number of amino acid substitutions, deletions, insertions and/or additions that may be made to SEQ ID NO: 3. Thus, the scope of the claim includes numerous structural variants, and the genus is highly variant because a significant number of structural differences between genus members is permitted. Although the specification states that these types of changes are routinely done in the art, the specification and claim do not provide any guidance as to what changes should be made. Structural features that could distinguish compounds in the genus from others in the protein class are missing from the disclosure. No common structural attributes identify the members of the genus. The general knowledge and level of skill in the art do not supplement the omitted description because specific, not general, guidance is what is needed. Since the disclosure fails to describe the common attributes or characteristics that identify members of the genus, and because the genus is highly variant, SEQ ID NO: 3 alone is insufficient to

describe the genus. One of skill in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus. Thus, applicant was not in possession of the claimed genus.

**Conclusions:**

**Claim 1:**

The claimed subject matter is adequately described. A rejection under the written description requirement should not be entered.

**Claim 2:**

The claimed subject matter is not supported by an adequate written description because a representative number of species have not been described. A rejection under the written description requirement, relying on the analysis set out above, should be entered.

#### **Example 14: Product by Function**

**Specification:** The specification exemplifies a protein isolated from liver that catalyzes the reaction of A  $\longrightarrow$  B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO: 3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

#### **Claim:**

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A  $\longrightarrow$  B.

#### **Analysis:**

A review of the full content of the specification indicates that a protein having SEQ ID NO: 3 or variants having 95% identity to SEQ ID NO: 3 and having catalytic activity are essential to the operation of the claimed invention. The procedures for making variants of SEQ ID NO: 3 are conventional in the art and an assay is described which will identify other proteins having the claimed catalytic activity. Moreover, procedures for making variants of SEQ ID NO: 3 which have 95% identity to SEQ ID NO: 3 and retain its activity are conventional in the art.

A review of the claim indicates that variants of SEQ ID NO: 3 include but are not limited to those variants of SEQ ID NO: 3 with substitutions, deletions, insertions and additions; but all variants must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO: 3. Additionally, the claim is drawn to a protein which **comprises** SEQ ID NO: 3 or a variant thereof that has 95% identity to SEQ ID NO: 3. In other words, the protein claimed may be larger than SEQ ID NO: 3 or its variant with 95% identity to SEQ ID NO: 3. It should be noted that “having” is open language, equivalent to “comprising”.

The claim has two different generic embodiments, the first being a protein which comprises SEQ ID NO: 3 and the second being variants of SEQ ID NO: 3. There is a single species disclosed, that species being SEQ ID NO: 3.

A search of the prior art indicates that SEQ ID NO: 3 is novel and unobvious.

There is actual reduction to practice of the single disclosed species. The specification indicates that the genus of proteins that must be variants of SEQ ID NO: 3 does not have substantial variation since all of the variants must possess the specified catalytic activity and must have at least 95% identity to the reference sequence, SEQ ID NO: 3. The single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity. One of skill in the art would conclude that

applicant was in possession of the necessary common attributes possessed by the members of the genus.

**Conclusion:** The disclosure meets the requirements of 35 USC §112 first paragraph as providing adequate written description for the claimed invention.

**Example 15: Antisense**

**Specification:** The specification discloses a messenger RNA sequence, SEQ ID NO: 1, which encodes human growth hormone. The specification states that the invention includes antisense molecules that inhibit the production of human growth hormone. The specification describes an art-recognized method of screening for antisense molecules that is called “gene walking.” Gene walking is said to involve obtaining antisense oligonucleotides that are complementary to the target sequence.

**Claim:**

An antisense oligonucleotide complementary to a messenger RNA having SEQ ID NO: 1 and encoding human growth hormone, wherein said oligonucleotide inhibits the production of human growth hormone.

**Analysis:**

A review of the full content of the specification indicates that the complement of SEQ ID NO: 1 is essential to the operation of the claimed invention. The general knowledge in the art is that any full-length complement of a target mRNA inhibits the function of the mRNA and is therefore an antisense oligonucleotide. Thus, one of skill in the art would view applicant’s disclosure of a coding sequence, with the statement that the invention includes antisense oligonucleotides, as an implicit disclosure that the full-length complement of SEQ ID NO: 1 is an antisense oligonucleotide.



It is generally accepted in the art that oligonucleotides complementary to a messenger RNA, including fragments of the full-length complement, have antisense activity when they match accessible regions on the target mRNA. Generally, the closer the complementary fragment is to full length, the greater the likelihood it will have antisense activity. In addition, oligos that retain complementarity to the Shine-Delgarno sequence usually have antisense activity.

The claim is drawn to the genus of antisense molecules that inhibit the production of human growth hormone encoded by SEQ ID NO: 1. There is a single species described with a complete structure, i.e., the full-length complement of SEQ ID NO: 1. In addition to the full-length complement, the genus includes fragments of the complement that retain antisense activity.

The procedures for making oligonucleotide fragments of the SEQ ID NO: 1 complement are conventional, e.g., any specified fragment can be ordered from a commercial synthesizing service. The procedures for screening for antisense activity are also conventional, and the specification describes the assay needed to do gene walking. The experience accumulated in the art with gene walking is that numerous regions of a target are accessible, that these regions are identified routinely, and that antisense oligonucleotides are complementary to these accessible regions. The full-length complement and longer fragments match multiple accessible regions; shorter fragments match fewer accessible regions.

When considering the distinguishing characteristics of the claimed invention, the sequence provided in the specification defines and limits the

structure of any effective antisense molecules. The specification also teaches the functional characteristics of the claimed invention as well as a routine art recognized method of making and screening for the claimed invention. Considering the specification's disclosure of:

(1) the sequence (SEQ ID NO: 1) which defines and limits the structure of any effective antisense molecules such that one skilled in the art would be able to immediately envisage members of the genus embraced by the claim, and

(2) the functional characteristics of the claimed invention as well as a routine art-recognized method of screening for antisense molecules which provide further distinguishing characteristics of the claimed invention, along with

(3) the general level of knowledge and skill in the art, one skilled in the art would conclude that applicant was in possession of the invention.

**Conclusion:** The claimed invention is adequately described.

### **Example 16: Antibodies**

**Specification:** The specification teaches that antigen X has been isolated and is useful for detection of HIV infections. The specification teaches antigen X as purified by gel filtration and provides characterization of the antigen as having a molecular weight of 55 KD. The specification also provides a clear protocol by which antigen X was isolated. The specification contemplates but does not teach in an example antibodies which specifically bind to antigen X and asserts that these antibodies can be used in immunoassays to detect HIV. The general knowledge in the art is such that antibodies are structurally well characterized. It is well known that all mammals produce antibodies and they exist in five isotypes, IgM, IgG, IgD, IgA and IgE. Antibodies contain an effector portion which is the constant region and a variable region that contains the antigen binding sites in the form of complementarity determining regions and the framework regions. The sequences of constant regions as well as the variable regions subgroups (framework regions) from a variety of species are known and published in the art. It is also well known that antibodies can be made against virtually any protein.

**Claim:** An isolated antibody capable of binding to antigen X.

#### **Analysis:**

A review of the full content of the specification indicates that antibodies which bind to antigen X are essential to the operation of the claimed invention. The level of skill and knowledge in the art of antibodies at the time of filing was such that production of antibodies against a well-

characterized antigen was conventional. This is a mature technology where the level of skill is high and advanced.

The claim is directed to any antibody which is capable of binding to antigen X.

A search of the prior art indicates that antigen X is novel and unobvious.

Considering the routine art-recognized method of making antibodies to fully characterized antigens, the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature, one of skill in the art would have recognized that the spectrum of antibodies which bind to antigen X were implicitly disclosed as a result of the isolation of antigen X.

**Conclusion:** The disclosure meets the requirement under 35 USC 112 first paragraph as providing an adequate written description of the claimed invention.

**Example 17: Genus-species with widely varying species**

**Specification:** The specification discloses the rat cDNA sequences for proinsulin and pre-proinsulin and a method for determining the corresponding human and other mammalian insulin cDNA sequences. However, the specification does not disclose any actual cDNA sequence other than the rat proinsulin and pre-proinsulin sequence. The specification discloses that one human proinsulin amino acid (but not cDNA) sequence was known at the time of filing. The art recognized that the sequence of human insulin proteins, and therefore also cDNAs, would probably vary among individuals. The specification also discloses that pre-proinsulin is post translationally modified to form proinsulin, and that proinsulin is cleaved to form insulin.

**Claims:**

Claim 1. An isolated mammalian cDNA encoding insulin.

Claim 2. The isolated cDNA of claim 1 wherein the mammalian cDNA is human.

**Analysis:** The examiner should analyze claim 2 first because it is drawn to a subgenus of the genus of claim 1.

**Claim 2:**

A review of the full content of the specification indicates that human cDNA molecules that encode insulin are essential to the operation/function of the invention.

Claim 2 is directed to a genus of human cDNA which encodes insulin.

There is no species of human insulin cDNA disclosed.

Based upon art published after applicant's filing date there is expected to be variation among the species of cDNA which encode human insulin because the sequence of human insulin proteins, and therefore also human insulin cDNAs, would be expected to vary among individuals.

The specification discloses only the sequence of a single human proinsulin protein, and does not disclose any human cDNA sequence at all.

In addition, there is no evidence on the record of a relationship between the structure of rat insulin cDNA and the structure of insulin cDNAs from humans or other mammals that would provide any reliable information about the structure of other insulin cDNAs on the basis of the rat insulin cDNA.

There is no evidence on the record that the disclosed rat cDNA proinsulin sequence had a known structural relationship to the human cDNA sequence, or to other mammalian cDNA sequences; the specification discloses only a single human proinsulin (protein) sequence; the art indicated that human proinsulin proteins were expected to be variable in structure; and there is expected to be variation among human cDNAs that

encode a given human proinsulin. In view of these considerations, a person of skill in the art would not have viewed the teachings of the specification as sufficient to show that the applicant was in possession of the claimed human cDNA.

**Claim 1:**

Claim 1 is directed to a genus of mammalian cDNAs which encode insulin. The specification evidences actual reduction to practice of the rat cDNA sequences for proinsulin and preproinsulin, but does not disclose any other cDNA sequences. The art indicates that there is likely to be substantial variation among the species within the genus of cDNAs that encode mammalian insulins because the sequences of the mammalian insulin proteins, and therefore the mammalian cDNAs, would be expected to vary among species.

The specification discloses a method for determining the corresponding human and other mammalian insulin cDNA sequences as well as the function of the claimed sequences. However, neither the specification nor the general knowledge of those skilled in the art provide evidence of any partial structure which would be expected to be common to the members of the genus. Moreover, there is post filing date evidence that indicates that there is a lack of a structural relationship between the rat insulin cDNA sequences and other mammalian insulin cDNA sequences. In view of the above considerations one of skill in the art would not recognize that applicant was in possession of the necessary common features or attributes possessed by members of the genus, because rat cDNA sequences are not representative of the claimed genus. Consequently, since applicant was in

possession only of the rat insulin cDNA and since the art recognized variation among the species of the genus of cDNAs that encode mammalian insulin, the rat insulin cDNA was not representative of the claimed genus. Therefore, the applicant was not in possession of the genus of mammalian insulin cDNAs as encompassed by claim 1.

**Conclusion:**

Claims 1 and 2 do not meet the written description requirement.



**Example 18: Process claim where the novelty is in the method steps.**

**Specification:** The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of  $\beta$ -galactosidase using the claimed method using a cytochrome oxidase promoter.

**Claim:**

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

**Analysis:**

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of  $\beta$ -galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

**Conclusion:**

The claimed invention is adequately described.

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## REVIEW

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Mutagenesis of proteins is of paramount importance for understanding the relationship of protein structure to function. The functional and structural roles of amino acid residues in a protein of interest can be studied by comparing the mutant protein carrying changes in amino acid residues to the wild-type protein. To obtain samples of a specific mutant protein, the mutant gene must be isolated or created. Before the era of site-directed mutagenesis (SDM),<sup>2</sup> the only way to obtain a mutation was to isolate naturally occurring mutants with phenotypic or selective screening. Because the rate of naturally occurring errors in DNA synthesis is very low, such screening was usually done after treatment with mutagenic agents. Moreover, even if a screening test was available, isolation of mutants that are lethal or that do not produce observable changes in phenotype was not possible (1). In this procedure, also, the position and the type of mutations in the mutants obtained were often distributed randomly in the gene of interest, which may not have been what the researcher desired.

Mutagenesis, now achieved by either polymerase

chain reaction (PCR) or non-PCR, has revolutionized the means by which mutants are obtained (2, 3). Mutations can now be created precisely at a specific residue with a specific codon change to produce the desired amino acid substitution (SDM), which has allowed alteration of any amino acid residue in a protein without extensive screening. Thus, the SDM approach is much more efficient, yielding desired mutations in 50–100% of the molecules produced, than that of phenotypic screening (<1%). When a set of random mutations over a region or the entirety of a gene is desired, the approach of random and extensive mutagenesis (REM, also known as targeted random, region-specific, or library mutagenesis) has been developed, and has been improved greatly in recent years. The introduction of PCR has made both DNA synthesis and DNA mutagenesis very efficient processes, compared to that using the thermolabile polymerase and the single-stranded DNA as the template (non-PCR).

In recent years, advances in mutagenesis methods have made mutagenesis technology on the whole exceedingly complex. Here we review some of the newer approaches to *in vitro* DNA mutagenesis methods in both the PCR and the non-PCR categories. In addition, some of the *in vivo* DNA mutagenesis methods are briefly described. The applications of these methods in SDM, REM, scanning mutation, and deletion have been discussed. This review is intended to guide investigators, novice and experienced alike, in selecting DNA mutagenesis protocols according to their needs.

## BASIC PROCEDURES OF DNA MUTAGENESIS

To mutagenize a gene is to synthesize enzymatically a new DNA, while in the meantime incorporating the

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<sup>2</sup> Abbreviations used: SDM, site-directed mutagenesis; REM, random and extensive mutagenesis; PCR, polymerase chain reaction; UDG, uracil DNA N-glycosylase; DMSO, dimethyl sulfoxide; LCR, ligase chain reaction; USE, unique site elimination; bp, base pair(s); *Taq-Taq*, *Pfu-Pfu*, and *Vent-Vent*, DNA polymerases; dI, deoxyinosine phosphoramidite, monophosphate; dITP-deoxyinosine triphosphate; dATP, dCTP, dGTP, or dTTP-deoxyadenosine, deoxycytosine, deoxyguanosine, or deoxythymine triphosphate; dNTP-dATP, dCTP, dGTP, and/or dTTP.

desired mutations into this newly synthesized DNA. Other than attempting to introduce mutations into the product, the methods or protocols for DNA mutagenesis are essentially the same as those for DNA synthesis or for other molecular biological manipulations, such as cloning, sequencing, and probe labeling.

Three essential components are required in an *in vitro* DNA synthesis reaction: the template, the primer, and the four dNTPs, i.e., dATP, dCTP, dGTP, dTTP, in the presence of the enzyme, the DNA polymerase. In *in vitro* DNA synthesis, as *in vivo*, the DNA is semiconservatively synthesized as a single strand, incorporating the building blocks, dNTPs, based on base-pairing, i.e., G with C and A with T, with the template strand. *In vitro*, a short single-stranded DNA, referred to as a primer, is always required to be annealed to the template strand DNA to initiate the new DNA strand synthesis. Thus, dNTPs are always added, base by base, to the 3'-end of the primer; thus, *in vitro* DNA synthesis is often referred to as the primer extension. The template DNA, whether double-stranded or single-stranded, provides the existing strand to synthesize a second, new strand of DNA. For this reason, the product of DNA synthesis by the enzymatic reaction is always double-stranded DNA, made of the original (template) strand and the new (product) strand. Buffers and various other conditions are also important to ensure the success and the fidelity of DNA synthesis.

There are two types of *in vitro* DNA synthesis modules: the thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR. Accordingly, the DNA mutagenesis methods can be classified into the thermostable polymerase PCR and the thermolabile polymerase non-PCR, in addition to the *in vivo* DNA mutagenesis methods.

#### Thermostable DNA Polymerase-Based PCR

PCR involves *in vitro* synthesis of a DNA product, the two ends of which are defined by the positions of the two designed primers. As described above, DNA synthesis *in vitro* always requires an oligonucleotide primer, usually 20–30 bp in length, complementary to the template. These primers can be synthesized chemically exactly according to the designed sequence. Since a small number of mismatches, e.g., 1–3 bp, are tolerable for primer binding to the template, mismatches can be incorporated as mutations precisely in the newly synthesized DNA. Other than mismatches introduced through the primer, the mutant DNA is reproduced exactly according to the wild-type DNA template. Because of its simplicity, the primer has become a convenient source for introducing mutations (for reviews, see 2, 3, 100). PCR undergoes multiple heating and/or cooling cycles (average 30 cycles), each having three phases: denaturation at about 95°C, annealing at about

55°C, and extension at about 72°C. At the denaturation phase, the template is denatured so that the two strands are separated, to allow the primers to bind or anneal to them. At the annealing phase, the two primers are annealed to one strand each. At the extension phase, the annealed primers are then extended according to the template strands. Many cycles are then repeated in a such way. Thus both the original templates and their products, which predominate after several cycles over the original templates, become further templates for subsequent rounds of DNA synthesis. In this way, templates are amplified exponentially by around 2<sup>20</sup>-fold (13) to yield linear double-stranded mutant PCR products. PCR DNA synthesis is thus often called the amplification of the template DNA. Since thermostable DNA polymerases (usually *Taq* DNA polymerase) remain active throughout the high-temperature cycling, they need only be added once, at the beginning of the multiple cycles.

Usually, a mutant gene fragment or an entire mutant gene is produced by the PCR. A mutant fragment can then be used to replace the wild-type gene fragment in a plasmid or an entire mutant gene can be subcloned into a vector, for mutant screening and characterization. Thus, most often the subcloning is required, except that in the case of the inverse PCR, an entire linear mutant plasmid can be produced and then simply can be circularized.

#### Templates

The template DNA used for the PCR can be double-stranded or single-stranded, circular or linear. For the single-stranded template, in the first cycle only one of the two primers binds to this single strand and gets extended to form the double stranded-DNA template for the subsequent cycles. However, double-stranded plasmid DNAs have become favored for thermostable polymerase-based PCR DNA synthesis for several reasons. In comparison to single-stranded DNAs, double-stranded DNAs are easier to prepare. Also, gene inserts are in general more stable with double-stranded DNAs. Mutagenesis efficiency (50–100%) is similar for double-stranded and single-stranded templates. This trend has been reflected by the fact that the most recent commercial kits are based on double-stranded templates (Table 1).

#### Primer/Template Annealing

To ensure successful DNA mutagenesis, general rules for designing a good primer should be followed. Particularly, a primer for DNA synthesis should be free of strong secondary structures, such as hairpins, stem-loops, or direct repeats. Mismatches in general should be placed in the middle of a primer rather than at the 5'-terminus, although with some methods, such as the

TABLE 1  
A Survey of Available Commercial Kits for SDM

Company, kit	Year	Method	Reference
I. Kits for single-stranded templates			
Bio-Rad, MutaGene II	1991	dUTPase selection	(148)
USB, T7-GEN	1991	Methyl-dCTP	(147)
Amersham, Sculptor	1994	Phosphorothioate analogue	(146)
II. Kits for double-stranded templates			
Stratagene, DoubleTake	1992	Solid-phase strand separation	(48)
Clontech, Transformer	1994	Unique site elimination	(11)
GIBCO-BRL, PCR SDM	1994	PCR and UDG cloning	(23)
New England Biolabs, Code20	1994	Unique site near the mutagenesis site	(174)
Pharmacia Biotech, USE	1994	Unique site elimination	(11)
Promega, Altered Sites II	1994	Antibiotic resistance repairment	(140)
Stratagene, Chameleon	1994	Unique site elimination	(11)
Stratagene, ExSite	1995	Inverse PCR	(90)

inverse PCR and the ligation PCR (will be described later), it is preferable to place the mutation at the 5'-termini of the primers. Mismatches at the 3'-terminus of a primer generally affect the rates of DNA synthesis, although exceptions have been reported (77).

To hybridize the mutagenic primer correctly and sufficiently to its template, the annealing temperature should be chosen carefully. In general, the annealing temperature is chosen to be a few degrees below the melting temperature ( $T_m$ , the temperature at which half of the oligonucleotides are annealed to templates) of an oligonucleotide. Without any mismatches, the melting temperature in degrees Celsius is estimated (98) simply as follows:  $T_m = 4 \times (\text{number of G and C bases}) + 2 \times (\text{number of A and T bases})$ . With mismatches, the annealing temperature should be reduced further according to the number of mismatches in the primer. Alternatively, the minimal magnesium concentration should be increased from 1.5 mM for a perfect match to 2.0 mM for 1 mismatch in a 23-bp oligonucleotide (1/23 mismatch length) (99). When a mismatch is 2 bp in a 24-bp oligonucleotide, Mg concentration should be increased to 3 mM; for a 9-bp mismatch in a 41-bp oligonucleotide, it should be increased to 4 mM.

#### Areas of Concern on PCR with Recommended Resolutions

PCR was applied to DNA mutagenesis almost immediately after its inception in 1985, and has become a very useful method for DNA mutagenesis. Several areas of concern, together with suggested methods of resolution, are discussed below.

*Increasing the sequence fidelity of PCR products.* Similar to the DNA synthesis by PCR, the main draw-

back of using PCR for DNA mutagenesis is the relatively high rate of sequence errors in PCR products, often creating undesired mutations in addition to intended ones. *Taq*, the most widely used thermostable polymerase, lacks the 3'-5' exonuclease activity that "proofreads" any errors caused by 5'-3' DNA polymerase during DNA synthesis. Amplification through many cycles therefore accumulates errors. Two other polymerases, *Pfu* and *Vent*, which carry 3'-5' exonuclease activity, have been shown to provide 6-15 times the sequence fidelity of *Taq* (14-16). Thus, since their introduction, *Pfu* and *Vent* have been preferred for PCR-based DNA site-directed mutagenesis (17-19). When *Taq* was combined with even a small amount of *Pfu* or *Vent*, the fidelity increased about twofold, presumably because of the introduction of proofreading ability (16, 20, 21).

The concentrations of magnesium ions, dNTP, and pH may also affect sequence fidelity (16). The number of cycles can be reduced to achieve high fidelity, although this decreases the final amount of PCR products. This paradox can be solved by using larger amounts of methylated templates, which are readily removed after PCR by digestion with *DpnI*, a frequent-cutting used restriction enzyme which cleaves only methylated DNAs (22-25). Thus the use of a large amount of template makes it possible to amplify a large amount of products in a small number of cycles.

*Increasing the size and yield of PCR products.* Until recently, only products smaller than 3 kb could be amplified routinely by PCR. Longer products are often needed in experiments involving DNA mutagenesis (24). To obtain long PCR products, several factors must be optimized. For example, shorter denaturation time,

smaller reaction volume, longer extension time, and different buffer compositions (e.g., high pH, additional denaturants) are often used (26–29).

More recently, a small amount of 3'-5' exonuclease (*Pfu* or *Vent*), used as a secondary enzyme in addition to *Taq* as the main enzyme, has been shown to increase the size of PCR products (20). This improvement is attributed to the ability of 3'-5' exonuclease to correct *Taq*-induced sequence errors, which are thought to stall further amplification of large products (20). On the other hand, when *Pfu* or *Vent* was used alone without *Taq*, neither could support the amplification of long PCR products (20, 30). This may be the result of their 3'-5' exonuclease activity being higher than their 5'-3' polymerase activity. Therefore, the use of *Taq* and *Pfu* in the optimal combination is ideal for producing large mutant DNAs (19, 31). Mammalian genomic DNAs as large as 22 kb and phage  $\lambda$ -DNAs as large as 42 kb have been amplified successfully with this improved technique (20, 29). Kits for long PCR products are now commercially available from several sources.

**Amplifying DNAs with secondary structures or GC-rich regions.** Problems with secondary structures and GC-rich regions in DNA templates usually are remedied effectively with the use of PCR, which uses temperatures higher than those used in the thermolabile polymerase method, performed at 37°C. However, sometimes strong secondary structures or high GC-rich regions can remain in template DNAs even at temperatures employed in a PCR. Relevant to this problem, alkaline and heat denaturation before PCR can significantly improve the amplification of these DNAs (32, 33). "Hot PCR," in which the routine annealing temperature is increased from 55 to 65–72°C, is also useful in overcoming impediments caused by secondary structures or GC-rich regions (31, 34). In addition, denaturants (1–20% DMSO, 5–15% glycerol, 5–20% formamide, Tween-20, and NP-40) (34–38), T4 gene 32 protein (38), and *Escherichia coli* single-stranded DNA-binding protein (7) are helpful in amplifying such difficult DNAs. Similarly, tetramethylammonium chloride at 0.01–0.1 mM (39) or 10–120 mM (40) is effective in facilitating the amplification of difficult DNAs. Moreover, nucleotide analogs, 7-deaza-2'-deoxyguanosine triphosphate (41) or deoxyinosine triphosphate (42), can be incorporated into secondary structures or GC-rich regions to weaken the bonding between G and C bases.

Longer extension time is also beneficial for amplification of difficult DNAs (43). Presumably, the rate of DNA synthesis is lower at GC-rich regions or secondary structures. Finally, *Vent* and *Pfu* seem to be more capable of amplifying DNAs containing GC-rich regions or secondary structures than *Taq* (37, 43 and our own observation), although the mechanism is not clear.

### Thermolabile Polymerase-Based Non-PCR

In thermolabile DNA polymerase-based DNA synthesis, the DNA template is denatured with alkali and/or heat and annealed with the primer at the room temperature. Usually, one primer is used in the thermolabile polymerase-based non-PCR methods and mutations are introduced through mismatches in the mutagenic primer. DNA templates can be single-stranded or double-stranded, although single-stranded templates are preferred in this method. In the case of double-stranded DNA, only one strand bound with the primer is used as the template strand and the other strand is not used at all. DNA synthesis is then carried out at 37°C with thermolabile polymerases, which would become inactivated at higher temperatures and thus are called thermolabile enzymes. Nucleotide bases are added to the 3'-end of the primer according to base-pairing with the template. Consequently, a single-stranded DNA, still linear, complementary to the template strand, circular, is produced (2). This product is then circularized. Thus the product of this type of DNA synthesis is a hybrid DNA duplex carrying one template strand and one newly synthesized mutant strand. This hybrid is then used to transform (for plasmid or phagemid hybrids) or infect (for phage hybrids) *E. coli* to segregate mutant DNAs from wild-type DNAs. If M13 phage mutants are obtained, they are subcloned into a double-stranded expression vector for protein studies. Because there is only one round of DNA synthesis, there is no amplification of the template. In addition, when denatured double-stranded DNA is used as the template, only one of the two strands is actually used for the synthesis of the mutant strand.

### Templates

With thermolabile polymerases, mutant DNAs are synthesized more readily with a single-stranded than a double-stranded template, since two denatured complementary template strands of DNAs may quickly re-anneal at 37°C, interfering with the primer annealing with the template strand.

To prepare single-stranded DNAs as templates, often a gene is cloned into an M13 phage vector or a phagemid vector, which contains an M13 or a f1 replication origin, respectively (44). This preparation is time-consuming. Also, larger DNA gene inserts tend to be spontaneously deleted from the M13 phage vector during the process of the preparation (45), although long inserts in this type of vectors have also been successfully mutagenized. The single-stranded DNAs obtained are then used as templates to synthesize a mutant strand, directed by a mutagenic primer.

Alternatively, single-stranded linear DNA templates can be prepared from linear double-stranded plasmid DNAs or from PCR products that are always linear (46).



The two strands are separated from each other by the biotinylation of one strand (47). This biotinylated single-stranded DNA can then be used as the template for mutagenesis. For example, Weiner and colleagues (48) used such a template to synthesize a portion of a new strand with a mutagenic primer; the remaining portion was synthesized with an upstream primer. These two portions were then ligated. The resulting complete linear mutant strand was then separated from the biotinylated wild-type strand and recircularized. This circularization was aided by a bridging oligonucleotide binding to the two ends. The second mutant strand was then used to form the double-stranded mutant DNA. Although the steps are multiple, this approach avoids the problems or inconvenience of M13 phage or phagemid vectors. A kit is also available (Table 1).

Single-stranded linear DNAs can also be obtained from double-stranded DNAs by an asymmetric PCR—a PCR in which only one primer, rather than two, is used; thus, only one strand is linearly amplified. Sometimes, one primer is used at a concentration much higher than that of the other primer to produce mostly asymmetric products along with a small amount of double-stranded products. This type of template has allowed mutagenesis of relatively large mutant DNAs (0.5 and 1.1 kb) (49, 50).

### *Thermolabile Enzymes*

*Escherichia coli* DNA polymerase, a 5'-3' polymerase, was the enzyme first used in the thermolabile polymerase-based DNA synthesis. However, this polymerase is also active in other ways, having 5'-3' exonuclease activity and strand-displacement (helicase) activity as well. These two additional activities are undesirable in DNA mutagenesis procedures: mismatches introduced by the mutagenic primer can be removed by 5'-3' exonuclease activity, and newly synthesized DNA containing mutations can be displaced by wild-type DNAs by strand-displacement activity. Klenow, a proteolytic fragment of *E. coli* DNA polymerase, is more efficient for DNA mutagenesis, since it does not possess 5'-3' exonuclease activity; nonetheless, it does retain strand-displacement activity.

In recent years, phage-derived enzymes, i.e., T4 DNA polymerase, T7 DNA polymerase and sequenases (derived from T7 DNA polymerase without the native 3'-5' exonuclease activity), have become the most frequently used enzymes for DNA mutagenesis, since they do not carry either extra activity.

### *Primer/Template Annealing*

The general rules for the designing of primers mentioned in the PCR section should be followed. In addition, the non-PCR method requires a good region of match at each end, usually 10–15 bases. When the 5'-

terminus of a primer has a strong anchorage to its template, the strand displacement caused by Klenow or other enzymes will be minimized (10).

Adequate and correct annealing of templates and mutagenic primers is crucial for the synthesis of mutant DNAs. In the thermolabile polymerase-based non-PCR, the DNA template and the primer are encouraged to anneal with each other through a continuous or stepwise decrease in incubation temperature to below the estimated melting temperature, which is often room temperature. Additional cooling of the DNA template and primer complex on ice improves subsequent DNA synthesis (8, 10). When DNA templates were initially denatured by boiling, the snap-cooling of denatured templates on ice was shown to produce a better yield of mutant DNAs than the stepwise cooling to 25°C and then to 4°C (11).

When relatively long primers (for example, as required by deletion or insertion mutagenesis) are used for DNA synthesis with a thermolabile enzyme at 37°C, these primers may bind to incorrect regions of the template, and thus not produce the DNA products intended. To avoid such mis-priming, Hofer and Kuhlein (12) added Klenow at 65°C to the annealed template and primer complex and allowed the DNA synthesis reaction to proceed for 5 min at this higher temperature before cooling it to 37°C and adding more Klenow. The correct mutant DNA was obtained by this unusual treatment. Presumably at 65°C, residual activity of Klenow was present, which correctly extended and locked the primer onto the template. DNA synthesis then continued successfully at 37°C.

### *Secondary Structures and GC-Rich Regions*

In the thermolabile polymerase-based non-PCR, alkali and/or heat denaturation are used to separate two strands in order to allow binding of the primer and to destroy the local conformation of each strand, so that the DNA synthesis can proceed. DNA regions that are rich in guanine (G) and cytosine (C) may hinder the separation of the two strands, whereas secondary structures may slow down or stop DNA synthesis. This type of structure is particularly abundant in mammalian and viral DNAs, and may even exist in DNA vectors, e.g., pBluescript. In one study, no mutant DNAs were produced until the same insert was switched from a pBluescript into a pUC18 vector (4). Such complex structural conformations thus can be an impediment to efficient DNA mutagenesis using thermolabile polymerases.

Among thermolabile enzymes, T7 DNA polymerase is more capable of overcoming secondary structures than T4 DNA polymerase (Bio-Rad Technical Bulletin 1625; 5). Sequenase version 2, derived from T7 DNA polymerase, produces two to five times as much mutant DNA as T4 DNA polymerase does in parallel experiments (5). Klenow, however, with its strand-displace-



TABLE 2  
Comparison of Two Types of *in Vitro* DNA Synthesis Used in Mutagenesis

	PCR	Non-PCR
Reaction temperature/duration	Cyclic, typically 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), extension (72°C, 1 min)	Constant at 37°C for 1 h
Strands synthesized	Two strands	Only one strand
DNA polymerase	Thermostable, such as Taq and Pfu	Thermolabile, such as Klenow, T4 DNA polymerase
Primers required	At least two, sometimes more, one of the two may be mutagenic	Usually just one as the mutagenic primer, sometimes an extra one as the selection primer
Fold of amplification	$10^2$ – $10^6$	<1
Product synthesized	Shorter, linear double-stranded DNAs, usually containing only the coding region of a gene	Longer, hybrid plasmid, phage or phagemid DNAs carrying linear mutant strand and circular wild-type strand
Sequence fidelity	Relatively low	Relatively high
Hindrance due to secondary structure GC-rich region	Not likely	Likely
Cloning	Inserted into a vector by ligation	Circularized by ligation

ment activity, is more capable of overcoming certain secondary structure problems than T7 DNA polymerases or sequenases (6). Additives such as *E. coli* single-stranded DNA-binding protein can be employed to overcome secondary structure problems (7). DMSO (8) and certain weak base-pairing deoxyguanosine or deoxycytosine analogs, used in DNA sequencing to overcome the so-called compression problem caused by secondary structures, may also be used to alleviate secondary structures in DNA mutagenesis (9).

In summary of the basic procedures of DNA synthesis or DNA mutagenesis, a comparison of various aspects of the thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR are listed in Table 2. Novice readers are advised to consult additional references (86, 13). In addition to the *in vitro* DNA synthesis (PCR and non-PCR), the *in vivo* DNA mutagenesis methods will also be described briefly in the subsequent section. Basically, the various methods or protocols for *in vitro* (PCR or non-PCR) and *in vivo* DNA mutagenesis are assembled by manipulating the DNA templates, the primers, the synthesis materials (dNTP), or the synthesis conditions to meet different kinds of mutagenesis needs, such as SDM, REM, deletion and nested deletion, insertion, and scanning mutations. To generate a mutant DNA, in some cases both *in vivo* and *in vitro* methods, or both thermostable polymerase-based PCR and the thermolabile polymerase-based non-PCR methods, are used in the protocol, thus making the classification less clear.

#### APPLICATIONS OF PCR IN DNA MUTAGENESIS: MUTANT-DNA CONSTRUCTION STRATEGIES

##### Site-Directed Mutagenesis

In SDM, a specific mutagenic primer is used, resulting in a specific mutant with a predetermined site

and type of mutation. Although an amino acid can be replaced by any of the remaining 21 amino acids, possibly even by some artificial amino acids (101), some substitution mutations may result in a drastic global conformational change of the protein or a mistargeting to its site of action. Such a change will almost certainly bring about a significant change in function. Therefore, for studies aiming to map out functionally important amino acid residues in a protein, changes in the residues that alter only the local conformation of a protein are generally desirable. Guidelines for preferred and avoided substitutions have been suggested for this purpose (102).

The following described methods or protocols are generally used for SDM and are classified according to the desired position of the mutations in the PCR product.

##### Introducing Mutations into the Terminus of a PCR Product

Mutations are simply introduced by one of the two primers, and thus mutations are located within approximately 30 bp of either end of a PCR product. A pair of restriction sites is also usually built into these two primers. The resulting PCR product can then be readily cloned into a vector with one or two compatible restriction sites (62).

##### Introducing Mutations into the Middle of a DNA (Connecting PCR)

*In vitro* ligation of two PCR products carrying mutations. To create mutations in the middle of a relatively large DNA, two separate PCRs can be performed to amplify two halves of a complete gene, using four primers (Fig. 1A). An outside-forward primer is paired

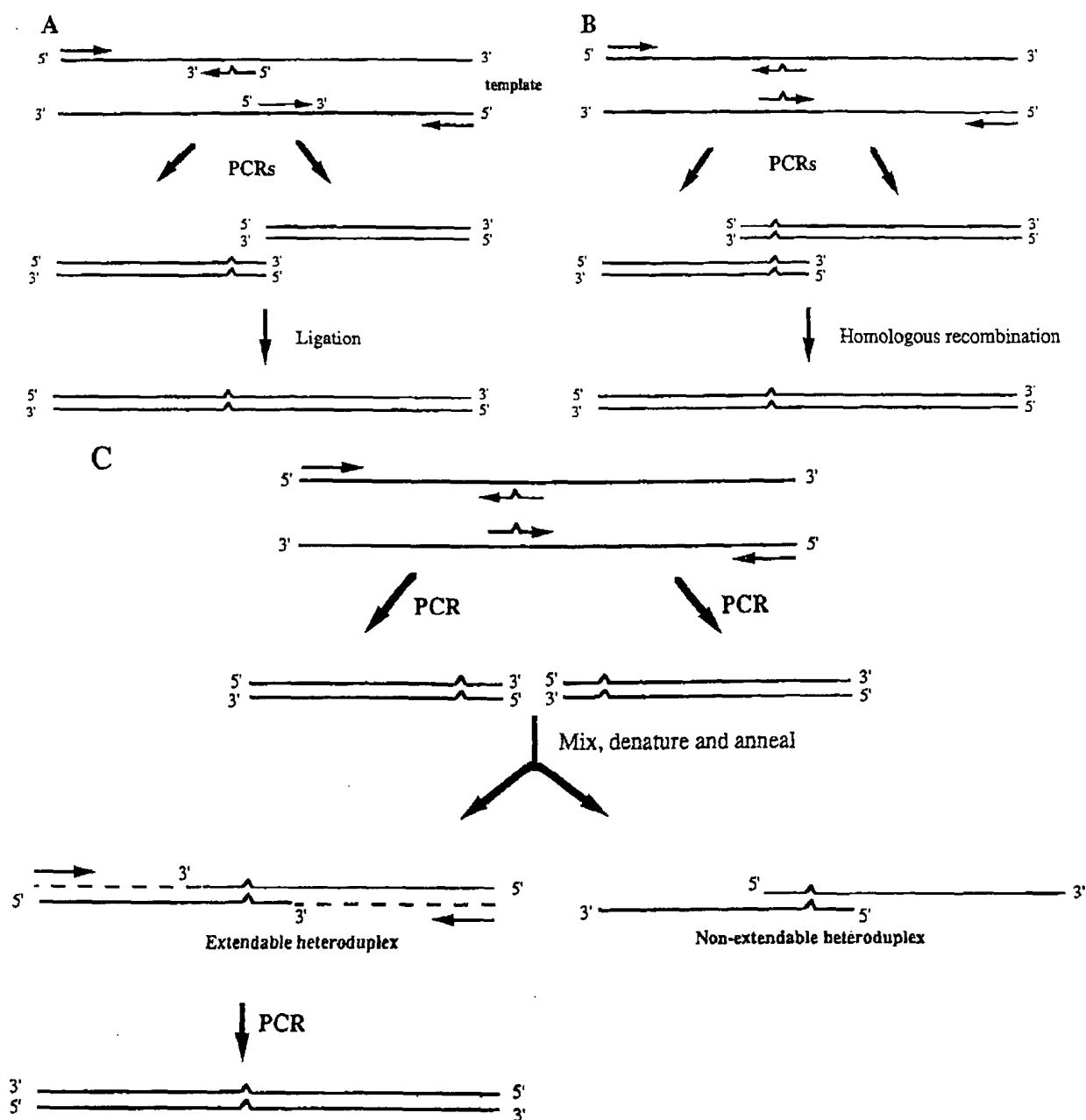


FIG. 1. Introducing mutations into the middle of a DNA (connection PCRs). (A) Introducing mutations by *in vitro* ligation of two PCR products. Two separate PCRs are performed to obtain two halves of a completed gene. The resulting two PCR products are then ligated to form a fused DNA carrying mutations situated in the middle and introduced by middle primers. (B) Introducing mutations by *in vivo* homologous recombination. Two PCRs are performed to generate two fragments carrying overlapping sequences introduced by two middle primers. The outside ends of these two fragments are ligated into a vector and transformed into the bacteria. As a result of the homologous recombination *in vivo* in the bacteria, one of the two overlapping sequences is removed and two fragments are joined together. (C) Introducing mutations by *in vitro* overlap-extension PCR. Two PCRs are performed to produce two fragments that carry overlapping sequences. These two fragments are then mixed, denatured, and annealed in a PCR-ready buffer to obtain mutant DNAs in a further PCR.

with a middle-reverse primer to synthesize the first half of the gene; an outside-reverse primer is paired with a middle-forward primer to synthesize the second

half. These two half-fragments which do not overlap are thus aligned in "tail-to-tail" fashion. The two resulting PCR products can be phosphorylated and then

ligated to form a fused DNA carrying mutations situated in the middle (60, 63–65). One or both of the middle primers can be mutagenic. This fused mutant DNA can be subcloned directly or can be amplified further using two outside primers before subcloning (66, 67).

Alternatively, a common restriction site can be built in at the 5'-terminus of both middle primers. The resulting two PCR products can be digested and then ligated. Sometimes, the incorporation of a restriction site into the middle primers causes an undesirable alteration to the corresponding amino acid sequence of a protein. To circumvent this problem, a sequence for a unique Class II restriction enzyme can instead be tagged onto the 5'-terminus of both middle primers (68, 69). These tag sequences are completely removed by digestion with a Class II enzyme.

Usually, ligation is carried out with thermolabile T4 DNA ligase. A thermostable *Taq* DNA ligase has also been used along with *Taq* DNA polymerase in a ligase chain reaction (LCR) (65). After two primary PCRs, the resulting two fragments are mixed with wild-type templates to undergo LCR, by which two fragments are connected. In a similar method (70), a phosphorylated mutagenic primer and two outside primers were used to perform PCR and LCR concurrently. A 900-bp mutant DNA was generated. Although the use of *Taq* DNA ligase simplifies the procedure, its low yield of final ligated product makes it less desirable. The desired full-length product is also often contaminated or mixed with partial-length by-products.

*In vivo homologous recombination of two PCR products carrying mutations.* Homologous recombination *in vivo* in bacteria is a DNA-joining event between two DNA ends carrying the same or very similar sequences (mutually overlapping sequences; Fig. 1B). As a consequence of this homologous recombination, one of the two overlapping sequences is removed; the two fragments are joined together in the bacteria. Two PCRs are performed to generate the two initial fragments carrying overlapping sequences introduced by two middle primers. Mutations are also introduced by two middle primers. To ensure the presence of mutation sequences in all or most recombinants, mismatches are best placed in both overlapping sequences, or in the portion of a middle primer that does not overlap with the other middle primer (23).

After two PCRs, both outside ends of these two fragments are ligated into a vector. This linear DNA is then transformed into bacteria to restore a circular functional plasmid via *in vivo* homologous recombination (23). Mutagenesis efficiencies around 50% may be obtained.

*In vitro overlap-extension PCR.* Overlap-extension PCR was developed almost concurrently by Higuchi and associates (70) and by Ho and colleagues (71). (Interest-

ingly, most subsequent papers only cited Ho *et al.*). Two PCRs are performed to produce two DNA fragments that carry overlapping sequences (Fig. 1C) and intended mutations, both of which are introduced by two mutagenic middle primers. Two PCR fragments are then mixed, denatured, and annealed in a PCR-ready buffer, to generate two heteroduplexes via overlapping sequences. Only one of the two heteroduplexes, which carries two 3'-termini at the joint, can be extended by *Taq* DNA polymerase to form a full-length double-stranded mutant DNA. The other heteroduplex, since it carries two 5'-termini at the joint, is not extendible. The extended double-stranded mutant DNA is amplified in a further PCR using two outside primers.

The limitation of this overlap-extension method is that when the intended mutant DNA is longer than 600 bp, or when the intended position of mutations is located near either end, the yield of desired mutant DNAs drops dramatically (72). Nonetheless, when an additional extension step and long PCR technology were used (31), it was possible to prepare mutant DNAs as large as 3.1 kb, even when GC content was over 60%.

#### *Introducing Mutations Anywhere in a Final PCR Product*

*The megaprimer PCR method.* Conventionally, primers or oligonucleotides are chemically synthesized, single-stranded, and relatively short. Then, it was observed that double-stranded DNAs of several hundred base pairs can also serve as primers for DNA synthesis or mutagenesis (5, 74). Because these primers are larger than conventional ones, they are called megaprimers.

In the megaprimer mutagenesis method, two outside primers, one middle mutagenic primer (reverse or forward, whichever direction makes the megaprimer smaller), and wild-type templates are needed (Fig. 2). The first PCR, using one outside primer, the middle mutagenic primer, and wild-type templates, is performed to form a double-stranded megaprimer containing mutations introduced by the mutagenic primer. In the earlier method (Fig. 2), this megaprimer is then purified and used together with the other outside primer and wild-type templates to obtain the final mutant product in a second PCR (35, 75). In a more recent modified method (Fig. 2), the purified or nonpurified megaprimer is extended by using wild-type templates (17). Only one strand of this double-stranded megaprimer is extendible to form a single-stranded full-length mutant template, which is then amplified by two outside primers in a second PCR to generate a large amount of double-stranded mutant DNA (35, 74, 76).

The template-independent addition of a nucleotide (most frequently adenosine) to the 3'-terminus of a product by *Taq* DNA polymerase is often seen in PCR

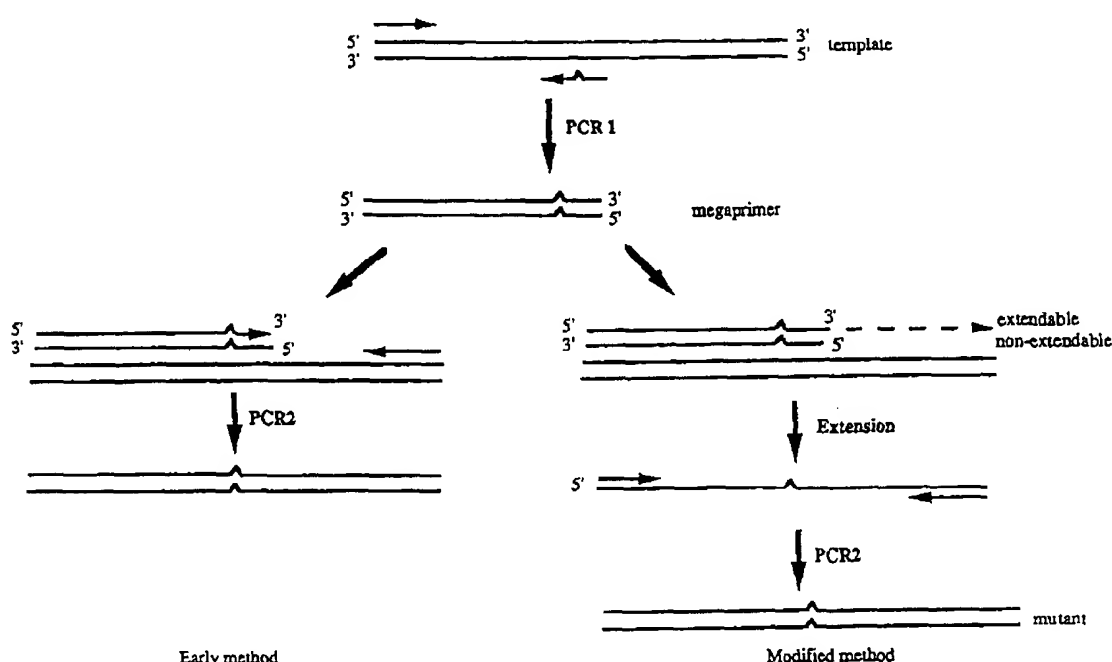


FIG. 2. The megaprimer method. A PCR is performed to form double-stranded megaprimer. Early method: This megaprimer is then purified and paired with an outside primer to obtain the final mutant product in a second PCR. Modified method: The megaprimer formed in the first PCR then is extended to form a single-stranded mutant template which is used to further amplify the final mutant product in a second PCR.

products. More often than not, this terminal addition is a mismatch for the megaprimer in relation to its template, thus stalling the extension of the megaprimer. Sometimes such 3'-terminal mismatches are tolerated (77), but the result is an unintended change to the amino acid sequence. Sharrocks and Shaw (78) suggested that the 5'-terminus of the mutagenic primer should be preceded immediately by a wobble base of a codon. Thus, in many cases, the addition of a mismatched base at the 3'-terminus of the megaprimer does not alter the amino acid sequence. Another interesting solution to this problem is to design the mutagenic primer in such a way that its 5'-end is immediately preceded by a T (79). In this way, any A added by the 3'-terminal transferase activity of *Taq* DNA polymerase does not interfere with the subsequent extension of megaprimers or change in amino acid sequence.

Alternatively, a universally applicable strategy is to attach a Class II enzyme-recognition sequence to the 5'-terminus of the mutagenic primer (80). Before the extension and the second PCR, the megaprimer formed from this mutagenic primer can be purified and digested with this enzyme, which removes residues of recognition sequences along with any 3'-terminal mismatches. Finally, both *Pfu* and *Vent* DNA polymerases, which lack 3'-terminal transferase activity, are superior to *Taq* for use in the megaprimer PCR method (17, 18).

Megaprimers, being long, double-stranded DNAs, are often difficult to denature, to anneal, and to extend to form full-length mutant templates. The megaprimer can be extended more efficiently by lengthening the times for the denaturation, extension, and (particularly) annealing in the extension step (19, 54, 81, 82), or by allowing additional cycles for the extension step (83). Several strategies are also used to selectively enrich the useful strand of the megaprimer, e.g., by performing an asymmetric PCR (19, 73) or by separating it from the other strand by biotin-labeling the corresponding outside primer used in the initial PCR (84).

*Improved megaprimer methods.* Picard and co-workers (18) attempted to combine the three stages of the megaprimer method. For the first 10 cycles of PCR, small amounts of both the mutagenic primer and one outside primer were added in a reaction tube to form the megaprimer. For cycles 11–20, a relatively large amount of the other outside primer was added, allowing the amplification of full-length mutant DNAs. For cycles 21–30, amplification was further encouraged by adding more of both the outside primers. Several interventions were required for this one-tube PCR, and product yields were low.

The megaprimer method recently has been improved further by the authors, so that it is now practical to form a large megaprimer with a high yield of final products (19). This makes it possible to mutagenize a large gene cassette without any unique restriction enzyme

sites in the region of interest. It is now also more convenient than before: the operator can now set up a three-stage PCR in one tube (much like a routine PCR) and allow programming software to run it automatically without any human intervention. All required components, including three primers (two outside and one mutagenic), are placed together with DNA templates and a DNA polymerase in one tube at the beginning of the PCR. The basis of this method is that each stage of this PCR is optimized specifically for a single function by manipulating conditions, such as the number of cycles, the concentrations of mutagenic primers and templates, and the use of a partial dideoxynTP 3'-end-blocked template. The product from the first stage is a megaprimer containing the mutation; from the second stage, a full-length mutant template; and from the final stage, a mutant DNA in sufficient amounts. This modified One-STEP (One-Step Three-stage Efficient PCR) method not only has made the megaprimer method more convenient, but also has increased the sizes of the megaprimer to at least 1.3 kb and the final mutant DNA to at least 5.4 kb, with high yields.

**The inverse PCR method.** Inverse PCR is commonly used for cloning unknown genomic sequences (e.g., 5'- or 3'-untranslated regions or introns) and has subsequently been used for sequencing unknown cDNA (89) (Ling and Robinson, manuscript in preparation). The inverse PCR method can also be used in mutagenesis (90), in which a pair of tail-to-tail primers is made from the site of the desired mutation and used to amplify the entire double-stranded plasmid containing the desired gene (Fig. 3). To introduce mutations, one of the two primers used is mutagenic. The resulting linear double-stranded PCR product is then circularized by ligating the two blunt-ends. To allow such a ligation, phosphate must be added to the 5'-terminus of the PCR products, by phosphorylating either the primers in advance or the PCR products afterward. The ligated DNA is then transformed into *E. coli*.

A unique restriction site may also be incorporated into both primers to create compatible cohesive ends in the PCR product, thus increasing the efficiency of circularization and removing the requirement for phosphorylation. In this case, if possible, either degenerate codons for amino acids that do not change the peptide sequence or a Class II enzyme sequence should be used (58). Alternatively, overlapping sequences can be generated at two ends of this linear double-stranded DNA through the two primers. The *in vivo* intramolecular homologous recombination in bacteria will circularize this product (91, 92). Instead of directly transforming this PCR product into bacteria, Jones and Winistorfer (93) have found that denaturation and re-annealing prior to transformation seemed to consistently yield 2- to 11-fold more transformants than di-

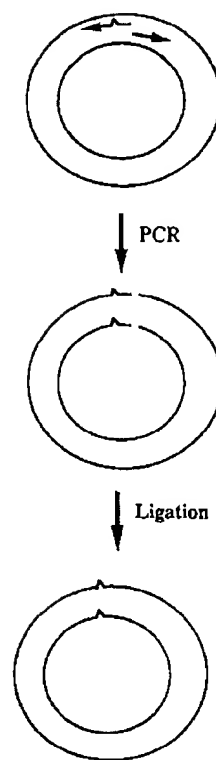


FIG. 3. The inverse PCR method. A pair of tail-to-tail primers is made from the intended mutation site and used in a PCR to amplify the entire plasmid containing the desired gene. This linear double-strand plasmid is then circularized and transformed into *E. coli*.

rect transformation. Likely, this treatment renders the two ends cross-annealed among the four strands, thus facilitating recombination *in vivo*.

Since it is often difficult to gel-resolve and purify the desired linear mutant PCR product from the circular wild-type plasmid template, the mixture of both is generally used to transform *E. coli*, resulting in a lower mutagenesis efficiency (92). The wild-type template should therefore be used in a low concentration (93–95). For example, the use of 100 ng plasmid DNA in the original method by Jones and Howard (91) resulted in a relatively low mutagenesis efficiency: less than 20%. However, when 10 pg to 1 ng DNA was used, more than 50% of transformants were mutant colonies (92). Another way to minimize wild-type background after transformation is to use alkali-denatured plasmid DNA templates (95, 96), which is beneficial probably because the alkaline treatment not only denatures the template more efficiently, but also makes the template less efficient in bacterial transformation. Coincidentally, Dorrell *et al.* (95) found that mutagenesis efficiency increased significantly when this pretreatment of templates was combined with the omission of phenotype recovery after the heat-shock step during *E. coli* trans-

formation. It is thus postulated that damage to wild-type templates caused by alkaline treatment is not repaired as a result of omitting the phenotype recovery step. Since newly synthesized mutant DNAs are not treated by alkali, they are thus not damaged.

This inverse PCR method is very simple and effective, and has been utilized to generate mutant plasmids as large as 11.5 kb (24), although it generally works best for plasmids under 3.1 kb.

#### Random and Extensive Mutagenesis

The REM approach is extremely useful when many mutations at a given position or many mutations at different positions are needed. For example, the REM approach has been used successfully in mapping out the important residues of proteins such as *Taq* DNA polymerase I (103) and *EcoRV* restriction endonuclease (104). The REM approach has also been used to engineer antibody specificity (60, 105) and enzyme specificity, such as triose-phosphate isomerase (106) and aspartase (107).

#### *Using Primers Randomly Spawned with Mismatched Bases or Ambiguous Bases*

In the REM, a set of mutagenic primers is generated in one synthesis reaction by supplying three mismatched bases at a single desired base position. This set of primers is then used to synthesize a set of mutant DNAs with three different mutations at the same position. Multiple sets of mutagenic primers can also be synthesized at once (one synthesis reaction) by providing a high concentration of the correct base and low concentrations of the three wrong bases at the multiple desired REM sites. Instead of supplying three mismatched bases for primer synthesis, deoxyinosine phosphoramidite (dI, an ambiguous base) may be added to a dNTP phosphoramidite reservoir at one or several base positions. The dI-containing primer does not specify the complementary base in the subsequent DNA synthesis. Since all four natural bases may pair with deoxyinosine, there is a 75% probability that any one of the three wrong bases, instead of a correct base, is paired with this ambiguous base. In some cases, trinucleotide phosphoramidite units representing codons for all 20 amino acids can be used to synthesize oligonucleotides. Those random mutagenic oligonucleotides with all possible amino acid mutations can be obtained quickly (108), although such oligonucleotide synthesis is too specialized to be useful to most investigators.

#### *Using Erroneous PCRs*

DNA synthesis *in vitro* tends to produce random sequence errors. This tendency is relatively great in the thermostable-polymerase PCR method, particularly

when *Taq* DNA polymerase is used. *Vent* and *Pfu* DNA polymerases make far fewer errors than does *Taq*. The error rate found by Zhou and colleagues (109)—that 35% of all subcloned PCR products synthesized by *Taq* contained at least one base substitution in a 633-bp product—is somewhat higher than that reported by Lerner and associates (110), who used a similar method.

Measures can be taken to increase the error rate of PCR for REM (1, 109, 111, 112). Factors that can be used to increase the error rate of a PCR include buffer composition (e.g., a high magnesium concentration, a high pH, or the addition of 0.5 mM  $MnCl_2$ ) and other conditions (e.g., a large amount of polymerase, a small amount of template, a great number of cycles, a long extension time, or a low annealing temperature). In addition, a biased pool of the four dNTPs, with concentrations differing by a factor of 10 to 1000, also encourages a higher error rate (111–114). Finally, the template may be treated chemically to encourage misincorporation in the DNA synthesis. Although the treatment itself alone is inefficient at inducing random mutations, it is effective when combined with an error-prone PCR (115, 116).

Under various combinations of these conditions, an error rate of 1 bp per 150 bp can be obtained per PCR. Thus, on average, every clone containing a gene of 150 bp is expected to have a single random mutation.

On the other hand, undesirable multiple mutations can occur when a highly error-prone PCR is used, which are caused by the compounding of mutations at various cycles. One possible solution to this problem is to amplify DNAs first in an error-minimized PCR for many cycles, and then in an error-prone PCR for only limited cycles.

A drawback of the PCR-based REM method is that different genes or different regions of the same gene tend to have different error rates, even when the same conditions are applied (unpublished observations from our laboratory, and 117). The resulting mutants also tend to have more transitions (A to G, or T to C) than transversions (A to C, T to G, A to T, or G to C).

#### *Using Deoxyinosine or Other Degenerate Base Analogs*

Deoxyinosine triphosphate or other ambiguous degenerate base analogs may be incorporated into PCR products when the analog is added to a mixture of three dNTPs at normal concentrations and the fourth dNTP at a lower concentration. This biased set of dNTPs forces dI to be incorporated in place of the fourth base during DNA synthesis. This dI-containing DNA then serves as a template for subsequent cycles of DNA amplification. Theoretically, there is a 75% probability that wrong bases may be incorporated wherever a dI

is located, since this dI complements weakly with all four natural bases (42). Spee and co-workers (118) found that using this method, up to 4 bp mutations per 1000 bp could be produced per PCR, which on average corresponds to a 100% mutagenesis efficiency for each clone containing a 250-bp PCR product. Under a different PCR setting, using 0.2 mM dITP in addition to 0.2 mM each dATP, dCTP, dGTP, and dTTP, Ikeda and colleagues (105) obtained 19 mutants carrying a single mutation out of 52 screened clones for a 300-bp PCR product.

PCR methods using dI or other degenerate base analogs seem to be less sequence-dependent than error-prone PCR methods do, and also seem to have less bias toward transitions against transversions (1).

### Scanning Mutations

Scanning mutagenesis or linker scanning mutagenesis is a technique designed to systematically change sequence segments in a regular increment (e.g., every 20 bp) into a common sequence (e.g., 2–5 bp) within a gene. Thus, a set of scanning mutant DNAs can be obtained to map out functionally important regions of a protein quickly.

An example is the so-called alanine scanning mutagenesis, in which amino acids at different positions of a protein are replaced systematically with alanine. The reason for choosing alanine as the common replacement amino acid is that it does not change the peptide-chain orientation, as do glycine and proline, and also it does not possess extreme steric and electrostatic characteristics (123). Cysteine can be used as a common residue as well to probe whether or not a region of a protein is membrane-spanning, since cysteine interacts with a readily detectable membrane-permeable thiol reagent, *N*-ethylmaleimide (124). A protease-cleavage site can also be incorporated as a common sequence, to identify quickly the corresponding mutant protein by protease digestion (125). In scanning mutagenesis studies of DNA structure and function correlation (e.g., promoter DNA), the common sequence usually contains a restriction site to facilitate the screening of mutant DNAs (126).

Overlap-extension or megaprimer PCR methods may be used to create such scanning mutants (127, 128).

### Deletions and Nested Deletions

Small deletions (less than 10 bp) can be made easily by an oligonucleotide primer design. For larger deletions, relatively long primers can be used, which loop out the sequences to be deleted. A 152-bp intron has been deleted successfully in this way by using a 30-bp primer (129). Larger deletions may also be produced by joining two PCR-amplified fragments, leaving out a portion of a DNA fragment (30).

Nested deletion of DNAs is another way to map out functionally important residues of a protein (130). Traditionally, a linear DNA can be deleted stepwise from one end by an exonuclease III digestion followed by a S1 nuclease digestion. Both the amount and the duration of enzyme digestions can be adjusted carefully to produce a set of nested-deletion DNA mutants. DNAs can also be labeled randomly with thio-dNTPs before the exonuclease III treatment. Since thio-containing sequences are resistant to exonuclease digestion, wherever the thio-dNTP is incorporated into the DNA, the digestion will stop. In this way, digestion can be controlled readily to obtain different incremental fragments (131). A more convenient and increasingly popular method is to amplify a set of nested deletions with a common primer from one end by PCRs, paired with a series of primers made by varying positions from the other end. Obviously, numerous oligonucleotides are required.

### Insertion

Small insertions (fewer than 10 bp) can be made easily by designing mutations into primers. Relatively long inserts should be attached to the 5'-terminus of a primer when used in an inverse PCR method. Long insertions can be made by the use of the megaprimer or an the overlap-extension PCR. An insertion sequence can also be placed in the middle of a relatively large primer for mutant strand synthesis (132). For insertions greater than 200 bp, three individual fragments, one of which is the inserting fragment, can be produced by PCRs and subsequently joined by ligation (30, 133).

### Multiple Mutations

Multiple mutations are sometimes required in the same gene for studies of whether a second mutation site modulates another (134). Multiple mutations can be obtained simply when several mutagenic primers are used one at a time for several rounds of mutagenesis (135). Sometimes, desired multiple mutations can be obtained even when all mutagenic primers are placed at the same time in the same reaction (136). Alternatively, a few DNA fragments, each carrying mutations, can be connected to generate a joined product with multiple mutations (65).

## APPLICATIONS OF PCR IN DNA MUTAGENESIS: MEASURES OF MUTANT SELECTION

### Nested PCR

For the megaprimer PCR method, the challenge is to amplify selectively the mutant template in the second PCR, without concurrent amplification of the coexisting wild-type template. A popular nested PCR strategy is



to use a tagged outside primer in the first PCR. This tagged sequence, 10–30 bp long, is located at the 5'-end of the primer and is absent in the wild-type template (Fig. 4A). The resulting tagged megaprimer is then extended to form a single-stranded tagged mutant template by using wild-type templates. Then a new primer (often called the nested primer), made mostly from this tagged sequence, is used in the second PCR. Since this nested primer recognizes the tagged mutant template, not the wild-type template, primarily mutant DNAs are amplified. This strategy thus has increased significantly the efficiency of the megaprimer method (65, 149).

Instead of a nested primer, a template different in sequence only at one end from the first wild-type template is used for the extension of the purified megaprimer to form a single-stranded mutant template (Fig. 4B). This pair of templates can be obtained by cloning the insert into two different vectors. Since one of the two outside primers used in the second PCR will not bind to this template with a different end, only the mutant templates will be amplified (150, 151). A similar approach is to use two templates, differing in the orientation of the insert relative to flanking vector sequences by cloning an insert into the same vector in two opposite orientations, or into two related vectors differing in only the orientations of cloning sites, such as pUC18 and pUC19. Thus, when one template is used for the first PCR and the other for megaprimer extension, without the purification (Fig. 4C), the resulting mutant template contains the same sequence on both sides and is therefore amplified in the second PCR by using only one outside primer derived from that sequence. In other words, this one primer functions as both a forward and a reverse primer; neither wild-type templates (orientation 1 or 2) will be amplified with this primer alone (152).

Steinberg and Gorman (82) and Liang and colleagues (99) capitalized on the use of two different templates, simply by using a piece of short DNA as a template for megaprimer extension (Fig. 4D). This short DNA, made by restriction digestion of the wild-type template or by PCR, supports the extension of the purified megaprimer but does not support the amplification of the full-length wild-type template in the second PCR (82, 99). To avoid the extension of this short DNA itself to form a full-length wild-type template, the 3'-end of this short DNA is blocked by adding dideoxynucleotides. Alternatively, this short template is made to carry an end nonhomologous to the megaprimer. In addition to using a short fragment for megaprimer extension, the template for the first PCR can also be a shorter fragment, lacking the opposite end of the megaprimer (153).

#### Purification of Mutant Products

Other than the above-described nested PCR, probably the most straightforward selection procedure for

PCR products is the purification of mutant DNAs from wild-type template DNAs by biotinylating one of the two primers for PCR (4). Thus, the newly synthesized mutant strand can be purified from wild-type templates. This biotinylated mutant strand alone can then be used as a template for the synthesis of the second strand. The resulting double-stranded mutant DNA can be circularized by digesting off the biotin cap with an enzyme, the site of which is built into the biotinylated primer (4). Finally, whenever the template and mutants are different in size, mutant PCR products may be purified from templates by simple gel electrophoresis.

#### APPLICATIONS OF PCR IN DNA MUTAGENESIS: SUBCLONING OF MUTANT PCR PRODUCTS

Most often, the mutant insert generated by PCR needs to be subcloned into an expression vector for protein studies. A recent review summarized several common ways to clone PCR products, including the purification of PCR products, the incorporation of restriction sites, the 3'-T-protruding vectors for cloning 3'-A-protruding products, and the cloning of blunt-ended products. Several aspects other than those discussed in Schaefer (157) are summarized below.

#### Recent Improvements on Ligation

Under the standard PCR conditions, the 3'-ends of some PCR products often are not extended completely; usually, they are missing the last several base pairs where restriction sites are designed through primers. Thus, PCR products often cannot be digested, leading to low efficiency or even failure of a ligation. An additional treatment of PCR products with *Pfu* DNA polymerase or T4 DNA polymerase combined with dNTP has been used to fill in the two product ends completely. Extra extension time (5–10 min, or even 60 min) at the end of a PCR process also helps to increase the efficiency of cloning (158).

Sometimes, when restriction sites are built too close to the end of a PCR product, those sites will digest with difficulty, even when the ends of those products are complete. Another alternative to increase the ligation efficiency is to create sticky ends in PCR products by partial digestion with exonuclease III.

It is also possible to create a sticky end without any digestion. The desired PCR product is amplified separately with two pairs of primers that differ only in that each primer in one pair is longer by 3 bp (e.g., GGG) at its 5'-end than that of the other pair. These two products are mixed in about equal molar ratio, denatured, and reannealed. Among all reannealed molecules, 25% will have 5'-GGG-protruding ends, and 25% will be 3'-CCC-protruding. Each molecule with sticky ends is then cloned easily into vectors



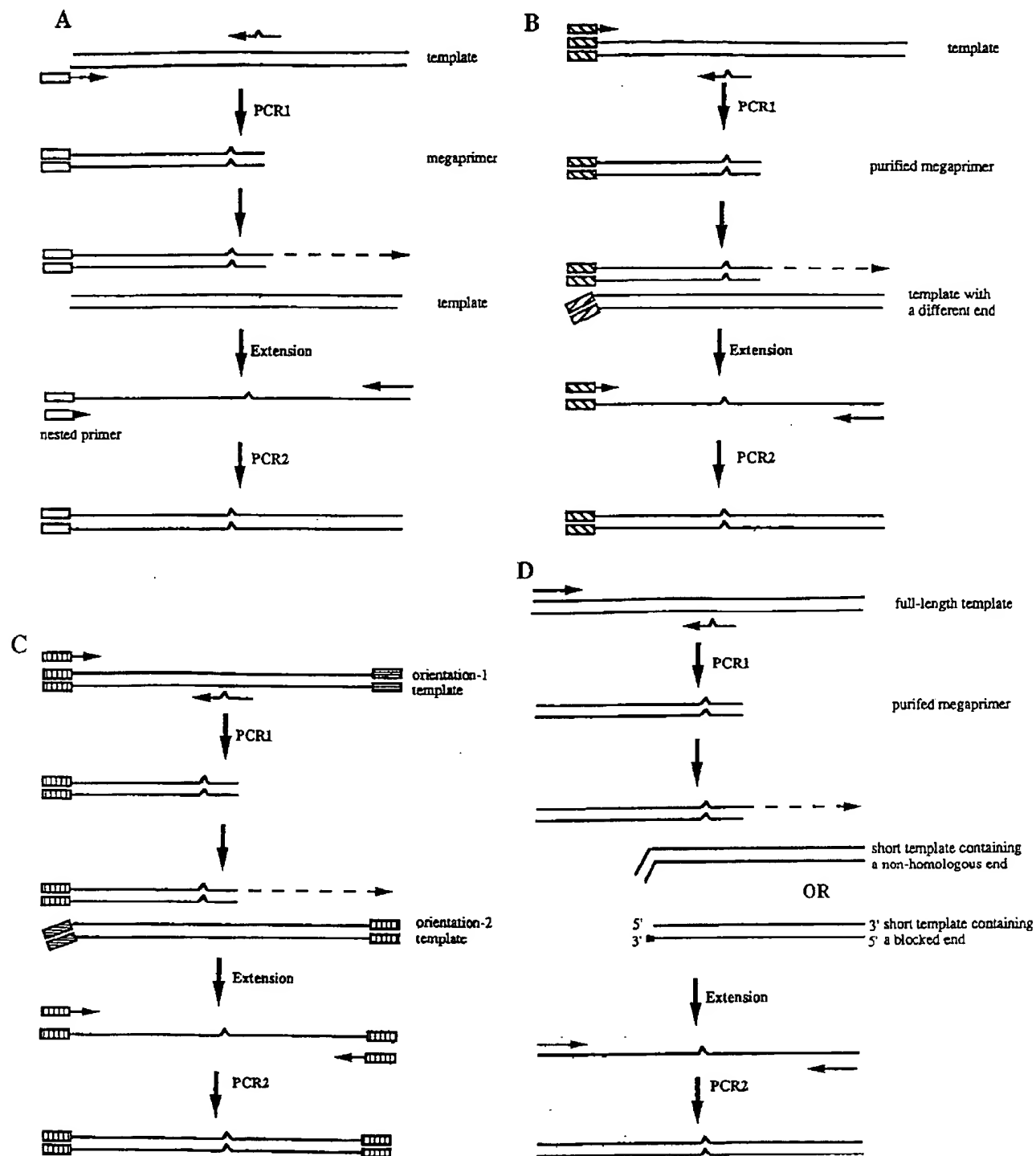


FIG. 4. Nested PCRs. (A) Nested primer. After the mutant template is formed, a nested primer (shown boxed and with an arrowhead) is used to selectively amplify the mutant template in the second PCR. (B) A different wild-type template. After the megaprimer is produced, it is extended on a template with a different end from the megaprimer, rather than the original template, to form the mutant template. The mutant template is then amplified selectively in the second PCR. (C) Two templates with different insert orientations. The megaprimer is produced by using the orientation-1 template in the first PCR, it is then extended on the orientation-2 template. This mutant template is amplified using only one outside primer. (D) Short template. After the megaprimer is amplified, it is extended on a short template. The mutant template is then amplified in the second PCR.

carrying compatible synthetic linkers. This nondigestion method gave significantly better cloning efficiency than TA cloning (159).

Ligation of vectors and inserts, both carrying blunt ends, is often difficult (160, 161). PCR thus may be used to amplify a product from the ligated molecule, carrying restriction sites that are easy to digest. Transformation of a secondary ligation would produce more of the desired clones (160, 161).

Usually, PCR products are purified from *Taq* polymerase by phenol and chloroform extraction, and from primers, dNTPs, and buffer components by gel electrophoresis and ethanol precipitation. However, even after these treatments, residual DNA polymerases and dNTPs may still be present in these PCR products, which modify restriction ends after the digestion. For example, 3'-recessive ends can be filled by *Taq* polymerase, and 3'-protruding ends can be removed by *Vent* or *Pfu*. Such alterations to the digested ends prevent subsequent ligation. Therefore, thorough extraction (three times) with phenol and chloroform is recommended by Benett and Molenaar (162). In some cases, even repeated phenol-chloroform extraction is not sufficient to remove *Taq* polymerase, which then must be degraded by a proteinase K digestion (163).

Primers, by default after their chemical synthesis, and hence PCR products, are usually not phosphorylated at their 5'-ends. Thus, vectors for cloning must be phosphorylated to allow the ligation of at least one strand between the vector and the PCR product. When both vectors and PCR products are phosphorylated, the efficiency of ligation increases greatly (164). Cyclic changes in incubation temperature between 10 and 30°C every minute also increase the ligation efficiency by 4- to 8-fold, compared to the constant temperature incubation (165). The transformation efficiency can also be increased greatly when the ligase is heat-denatured and products of the ligation reaction are purified from the buffer (166).

#### Ligation-Free Methods

PCR products may be inserted into a vector *in vivo* via homologous recombination in *E. coli* (167-169). Two primers used for the PCR are each attached with an overlapping sequence derived from the vector. As a result, each end of this PCR product has an overlapping sequence, which is homologous to its vector end. The vector and the insert are then transformed into a bacterial host and connected directionally via intermolecular homologous recombination. The minimal length of this kind of overlap sequence seems to be around 23 bp (168, 169). *In vitro* predenaturation and reannealing prior to transformation have been shown to increase this cloning efficiency (93). Long sticky ends (usually 12 bp or greater) compatible between a vector and an

insert can also be created by choosing or designing restriction enzyme ends in the vector which are made of only three nucleotide monophosphates in their terminal 12 bp or longer sequences. Treatment with T4 DNA polymerase in the presence of the fourth dNTP, and the absence of the three other dNTPs, will then remove nucleotides from one strand of the chosen terminal sequence, creating sticky ends. Both the insert and the vector are treated separately, creating terminal sequences that overlap. This cloning-free method is very efficient: Almost 100% of transformants have been found to be desirable clones (170).

Finally, primers can be designed to be rich in dUMP. The resulting PCR product, which contains dUMP at its ends, can then be digested with UDG, leaving 3'-protruding ends of about 12-15 bp. This fragment can be cotransformed into *E. coli* with vectors, carrying compatible 3'-protrusions made by using synthetic linkers. The yield of transformants of this method is high (171, 172), although dUMP-containing primers sometimes failed to amplify PCR products when *Pfu* and *Vent* were used (173).

#### APPLICATIONS OF *IN VITRO* NON-PCR AND *IN VIVO* METHODS IN DNA MUTAGENESIS

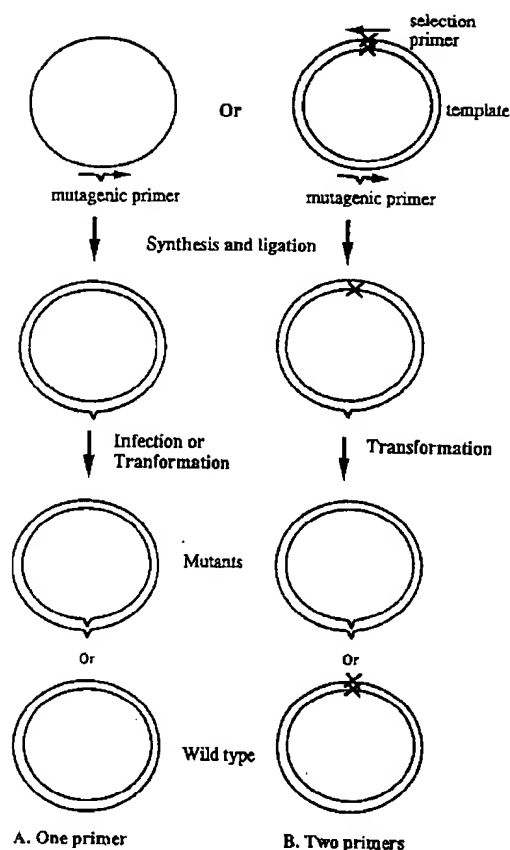
##### Mutant-DNA Construction Strategies

##### *Assembling Double-Stranded Oligonucleotides Carrying Mutations*

The simplest strategy for mutant DNA constructs of small size is to assemble a double-stranded DNA with a pair of cross-annealed oligonucleotides carrying desired mutations. This constructed DNA can be used to replace the wild-type counterpart by using naturally existing or artificially created restriction sites (55, 56). It is important that these sites be created in such a way that their creations do not unintentionally affect the sequence (57). Because most amino acid residues in a protein are encoded by multiple codons, it is possible to alter the DNA sequence to accommodate a restriction site without changing the amino acid sequence (58).

Several phosphorylated, mutually complementary oligonucleotides can be used to assemble a long double-stranded DNA (2, 59). Their neighboring ends can be ligated directly. If singled-stranded gaps remain after ligation, they can be filled in by a thermolabile DNA polymerase or alternatively by PCR, and then ligated with each other (60). Mutations are built in by swapping one of the numerous oligonucleotides with a mutagenic one (61).

Although simple, this procedure is limited to relatively short DNAs, generally 200-300 bp, otherwise too many oligonucleotides are required, making the procedure expensive. This strategy can be used to the applications of SDM and REM.



**FIG. 5.** The hybrid method. The mutant strand (the second strand), directed by the mutagenic primer (A, one primer) and by a selection primer (B, two primers), is synthesized using either single-stranded templates or denatured double-stranded templates under the catalysis of a thermolabile polymerase, to form a hybrid DNA carrying a wild-type strand and a mutant strand, which is then introduced into the bacteria to segregate the mutant and wild-type DNAs.

### The Hybrid Method

In this classical mutagenesis method, a circular single-stranded M13 phage DNA or phagemid DNA containing the desired gene is used as the template (2, 3, 85, 86). The mutant strand (the second strand), directed by a mutagenic primer, is synthesized under the catalysis of a thermolabile DNA polymerase, such as Klenow or T4 DNA polymerase, or T7 DNA polymerase (Fig. 5A, one primer). The second strand is then sealed by T4 DNA ligase. The resulting double-stranded DNA, a hybrid carrying a wild-type strand and a mutant strand, is then introduced into an *E. coli* to segregate the mutant and wild-type DNAs.

Using this method, wild-type DNA background levels as high as 99.9% are often encountered after transformation, for three reasons. First, the circular single-stranded DNA template for mutant-strand synthesis is often contaminated with short DNA fragments; these

can also, like regular primers, initiate DNA synthesis, producing wild-type double-stranded DNAs that are co-transformed with the hybrid into *E. coli* (87). Second, the sequence in the mutagenic primer may be displaced by the strand-displacement activity of Klenow polymerase, resulting in high wild-type background (2). Third, the host-directed DNA mismatch-repair system in bacteria usually favors methylated DNAs synthesized *in vivo* over nonmethylated DNAs synthesized *in vitro*. Thus, mutations in the synthesized mutants tend to be removed within *E. coli* (88). It is therefore often necessary to screen a large number of clones (85).

With the introduction of selection measures (two primers), the hybrid method using single-stranded DNAs becomes more efficient. Moreover, denatured double-stranded plasmid DNAs can also be used as templates to synthesize hybrid DNAs (Fig. 5B, two primers, discussed below). However, double-stranded DNAs are less efficient than single-stranded DNAs, since there is strong competition between binding of the complementary strand and the binding of the primer to the template. This method is used mostly in the SDM.

### Gapped Circles

A plasmid DNA containing a desired gene can be converted to have both single-stranded and double-stranded regions. This single-stranded region is for easy binding of a mutagenic primer. These so-called gapped circles are prepared by manipulating two DNA restriction fragments, both derived from the same plasmid. As outlined in Fig. 6, the combination of fragments I and II, of fragments I and III, or of fragments I and IV gives different pairs of gapped circles. Fragment I is made to lack the lower portion of the plasmid, which is the gene insert region, and usually is 10–40% of the whole plasmid. Fragment II is made to lack the upper portion of the plasmid and overlap with fragment I at both ends. Fragment III is a complete circular plasmid. Fragment IV is a complete linear plasmid obtained by a restriction enzyme digestion. Two fragments, in the above-mentioned combinations, are mixed, denatured, and reannealed to form a pair of gapped circles.

Depending on the designed primer, only one in each pair of gapped circles is the desired form as the template, containing a single-stranded region to which the mutagenic primer is complementary. This desired form can be gel-purified from the other form. For easier gel purification, the "gap" is better selected to be less than 12% of the total size of a plasmid (51). If a restriction site exists in the single-stranded region, the undesired gapped circle can be removed by enzyme digestion using an oligonucleotide to restore the full restriction site (12). The desired gapped circle alone can be produced by overlapping a 5'- or 3'-sticky restriction end of frag-

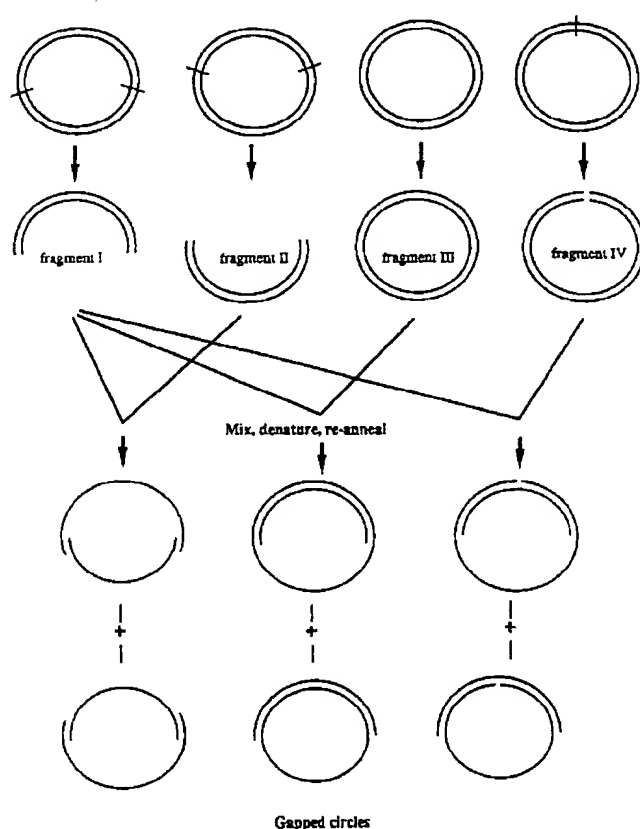


FIG. 6. The formation of gapped circles. A pair of restriction fragments is made from a plasmid; fragment I always lacks the region containing the intended mutation site. One of the other three types of fragments shown, containing the region missing in fragment I, is paired with fragment I. The two fragments in any of the three combinations shown are then mixed, denatured, and reannealed. Two forms of gapped circles are produced from each combination.

ment I with a compatible end of the second fragment (fragment II, III, or IV in Fig. 6) (52). Thus, two other strands carrying recessive restriction ends do not overlap to form the unwanted gapped circle.

The preparation of gapped circles in general is cumbersome. The purified gapped circle usually is not stable; thus, repeated preparations are unavoidable. The efficiency of mutagenesis using a gapped circle as a template with the thermolabile polymerase is relatively low, 5–15% (51); the gapped circle is no longer widely used. When certain selection measures are used together with this template (52), the efficiency can be increased (12, 53).

#### *In Vivo Mutagenesis Methods*

**The "closing" oligonucleotide method.** When a unique restriction site happens to be near the intended mutation position, the plasmid DNA can be cleaved at this unique site. A "closing" oligonucleotide primer

carrying mutations can be provided to cover the cleavage site. The linearized plasmid, together with this closing oligonucleotide, is cotransformed into bacteria for *in vivo* reconstitution (repairing). Only the repaired plasmid will survive in bacteria. Some of the repaired plasmids thus will contain mutations provided by closing primers (97).

The minimal length of such an oligonucleotide to seal the cleavage of a plasmid seems to be 20 nucleotides. The mutation position should be within 5 bp from the cleavage site, to obtain >15% mutagenesis efficiency. The farther the mutagenesis site from the cleavage site, the lower the efficiency. This method can be applied in the SDM.

**Using bacteria with defective DNA repair systems.** The error rate of DNA synthesis in a normal strain, typically  $10^{-10}$  errors per base per generation (119), is obviously too low to be used for DNA mutagenesis. DNA repair systems individually rendered defective, e.g., *mutS* (responsible for mismatch repair), *mutD* (responsible for sequence-proofing through 3'-5' exonuclease activity), or *mutT* (responsible for hydrolyzing oxodeoxyguanosine triphosphate) (120, 121), have been shown to increase significantly the spontaneous *in vivo* mutagenesis. When *mutS*, *mutD*, and *mutT* were rendered defective simultaneously (122), the spontaneous mutagenesis rate in the resulting bacterial strain (XL1-Red) was increased as much as 5000-fold over that in the wild-type strain, which means one mutation occurred in a 2000-bp DNA after 12–18 h of replication. On average, every clone containing a 2-kb gene would contain a mutation, and thus a plasmid containing the gene can be introduced into a such strain to produce random mutations. Although this rate of random mutagenesis is still low compared to that of PCR-based REM methods, the simplicity of this approach is very attractive to create the REM.

#### **Measures of Mutant Selection**

In most non-PCR methods catalyzed by thermolabile DNA polymerases, a significant amount of wild-type DNA is used as templates, which often remain with mutant DNAs and are cotransformed into *E. coli*. Thus, selection measures become the key to the success of mutagenesis. To support the growth of a bacterial host in an antibiotic-containing medium, a plasmid must have a functional antibiotic resistance gene and an intact replication origin, and must be in circular form. Circular plasmids are introduced into bacteria much more efficiently than linear ones. Even if a linear plasmid is introduced into bacteria, it has to be circularized to be replicated; only about 0.1% is recircularized *in vivo*. These characteristics have become the basis for the measures of mutant selection.

*Selections Based on Restoration of a Functional Antibiotic-Resistance Gene or Replication Origin of a Mutant Plasmid*

In this often-used method of selection, a plasmid in which the antibiotic-resistance gene is rendered defective (e.g., missing 1 bp) is used as a template to synthesize a hybrid plasmid, with a selection primer (a oligonucleotide to correct the defective sequence of the antibiotic-resistance gene) and a mutagenic primer (Fig. 5B, two primers) (137). As a result, only *E. coli* harboring the newly synthesized DNA carrying the selection primer sequence will survive on the antibiotic medium. To ensure that both primers are simultaneously incorporated, the mutagenic primer should be made to bind to the template preferentially. For example, its melting temperature should be higher than that of the selection primer. Both primers should also be in large molar excess (several hundred-fold) relative to template DNAs. Two resistance genes can also be used in the same plasmid, one for mutagenesis selection and the other for routine selection (138, 139). A mutagenesis efficiency of about 85% has been obtained by using this selection (138, 140).

Similarly, a selection measure can be based on the restoration of a defective replication origin (141). A phage or plasmid DNA in which the replication origin is rendered defective (e.g., missing 2 bp) can be used as a template to synthesize a hybrid plasmid in which a mutagenic primer and a selection primer (a oligonucleotide that restores the defective sequence) are incorporated into the mutant strand. Thus, only restored plasmids can be replicated in a normal *rnhA*<sup>-</sup> strain.

*Selections Based on Mutant DNAs Resistant to Restriction Digestion*

**Unique site elimination (USE).** Wild-type plasmids containing a unique restriction site are used as templates to synthesize a hybrid plasmid with a mutagenic and a selection primer (Fig. 4B). Mismatches are introduced into this hybrid plasmid by mutagenic primers to destroy this unique restriction site. Hybrid plasmids are then transformed into an *E. coli* strain with a deficiency in correcting mismatches (*mutS*<sup>-</sup>). Plasmid DNAs extracted from pooled transformants (a mixture of wild-types and mutants) are then digested with this unique site enzyme. Since mutants do not carry this unique site, they will not be digested by this enzyme, unlike the wild-type plasmids (5, 135). The resulting indigestible plasmid is then used to re-transform a *mutS*<sup>+</sup> *E. coli* strain (11, 123, 142).

In the original method (11), approximately 2 fmol of templates was used. It has been shown subsequently that if more template (5–25 fmol) was used, more mutant clones could be made (143). Because of the increased efficiency, competent cells treated by CaCl<sub>2</sub>

(10<sup>6</sup>–10<sup>7</sup> cfu/mg DNA) instead of highly competent cells (10<sup>8</sup>–10<sup>9</sup> cfu/mg DNA) may be used for the transformation. This is very useful because CaCl<sub>2</sub>-treated competent cells are easy to prepare and use, compared to highly competent cells. If a unique site is relatively close to the desired mutagenic position, two primers, selection and mutagenic, may be combined into one long primer, which is usually made by the PCR (5, 11, 135). Although the USE method and its modifications are almost universally applicable, two rounds of transformation are required. Some restriction sites, such as *Bam*HI, *Bgl*II, and *Pvu*I, do not work well for this selection (as outlined in the booklets from the Chameleon kit, Stratagene, and the USE mutagenesis kit, Pharmacia, and experience from our lab). When the size of the plasmid is larger than 6 kb, the USE method becomes less efficient.

**Digestion-resistant dNTP analogues.** Mutant strands can also be distinguished by replacing one or more of the four deoxynucleotide triphosphates with their 5'-[ $\alpha$ -thiol]triphosphate analogs during DNA synthesis. Synthesized hybrid plasmids are then treated with a restriction enzyme. Since wild-type strands contain only non-thiol sequences, they will be digested. Digested DNAs are removed by a further T7 exonuclease or exonuclease III treatment. The remaining mutant strand is then used as a template to synthesize a second mutant strand (53, 144). This selection can be used in conjunction with gapped-circle templates (53, 145, 146). A similar way of achieving this strand discrimination is to synthesize the mutant strand with methylated nucleotide triphosphate. The resulting fully methylated mutant strand will not be digested by certain frequent-cutting restriction enzymes such as *Msp*I, *Sau*3AI, and *Hha*I, but the nonmethylated wild-type strand will be digested (12, 145–147).

*Selections Based on Template DNAs Susceptible to Restriction Digestion*

Another powerful selection is to prepare DNA from *dut*<sup>-</sup> *ung*<sup>-</sup> bacterial strains, which do not restrict dUMP in DNAs, as do *dut*<sup>+</sup> *ung*<sup>+</sup> strains. Therefore, the resulting template DNA contains deoxyuracil (148). Since nascent mutant strands do not carry deoxyuracil, an ensuing digestion with the UDG enzyme exclusively eliminates template strands. Double-stranded mutant plasmids can then be made from the single-stranded mutant DNAs. Alternatively, the hybrid DNA can be transformed into a *dut*<sup>+</sup> *ung*<sup>+</sup> strain to eliminate the wild-type strand and to form double-stranded mutant DNAs. This selection method is often called the Kunkel method.

The template can also be prepared from a *Dam* methylase-carrying *E. coli*, which extensively methylates DNAs. The mutant strand synthesized *in vitro* is not

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TABLE 3  
Comparison of Major Methods of *in Vitro* Mutagenesis

	Duration (day) <sup>a</sup>	Man. time (h) <sup>b</sup>	Advantage	Disadvantage
Connection PCR <sup>c</sup>	6	9-10	Good efficiency, fast	Relatively shorter mutant products
Megaprimer PCR <sup>d</sup>	5	6-9	High efficiency, fast, larger mutant products	Needs careful setup of parameters and conditions
Inverse PCR	5	6-9	High efficiency, fast, simple, mutant product already in a plasmid	Needs to amplify long PCR products
Hybrid <sup>e</sup>				
Without selection	5-8	15	Mutant product already in a plasmid	Very low efficiency, lengthy, laborious,
With selection (USE)	7	12-14	High efficiency	Lengthy, laborious, some unique sites not suitable for this method
Gapped circle	7	13-15	Mutant product already in a plasmid	Relatively poor efficiency, lengthy, laborious, complex

<sup>a</sup> Duration is the total time from the beginning to the confirmation of mutant clones by sequencing, including waiting time (e.g., *E. coli* growth overnight).

<sup>b</sup> Man. time (manipulation time) is the time the operator needs to spend on performing all steps of that procedure.

<sup>c</sup> Connection PCR includes ligation, homologous recombination, and overlap extension of two PCR products to form the mutant product.

<sup>d</sup> Only the One-STEP version (19) was used for evaluation.

<sup>e</sup> Both methods with and without selection were evaluated. Without selection, the method needs time-consuming screening with radioactive selective hybridization (85).

methylated. When the hybrid plasmid is digested with a methylation-dependent enzyme such as *DpnI*, methylated wild-type template strands are removed selectively (24, 48).

#### Screening of Mutants

For the ease of screening of mutants made from either PCR or non-PCR methods, one of the primers should be incorporated with a sequence having a unique or rare restriction site (2, 10, 154), provided that this sequence does not bring about unwanted changes in the corresponding amino acid sequence. The desired mutations ultimately should be confirmed by sequencing. Random mutations occur even in non-PCR methods (155). For example, when T4 DNA polymerase was used in mutant DNA synthesis (5), 4 bp errors occurred in 6099 bp.

In summary of the sections on the applications of PCR and non-PCR/*in vivo* methods in DNA mutagenesis, some aspects of all major methods are compared in Table 3.

#### SUMMARY

In the last several years, the use of double-stranded DNA templates together with thermostable-polymerase PCR has essentially replaced the use of single-stranded DNA templates using thermolabile polymerase for *in vitro* mutagenesis. Numerous PCR methods are now available, such as overlap-extension PCR, megaprimer PCR, and inverse PCR. All of these PCR methods are reliable, effective, and convenient, al-

though they are more prone to high rates of spontaneous error in mutant DNAs than are methods using thermolabile polymerases. Some improvements, such as the introduction of methylated templates, have been employed to minimize PCR errors. On the other hand, because of the introduction of many selection measures (e.g., restoration of antibiotic resistance, restoration of replication origin and unique site elimination), both double-stranded and single-stranded DNAs can now be used as templates for mutagenesis using thermolabile polymerase methods. For PCR methods, selection measures such as nested PCR have been developed. All these selection measures have greatly improved the efficiency of mutagenesis by removing wild-type templates prior to transformation. Many efficient methods are available for both SDM and REM. Mutations can be introduced *in vitro* or *in vivo*, either by mutagenic primers or by erroneous DNA synthesis. Thus, choices largely depend on the experimental needs and resources of the investigator.

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